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(54) Title: INSULIN PRODUCING CELL-LINE

(57) Abstract: The invention provides insulin secreting human pancreatic cell lines and a process for their production from the electrofusion of normal human islet cells with immortal human cell lines to generate hybrid fusion cells which are able to produce insulin. The invention also provides uses for cell-lines.

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1 **"Insulin Producing Cell-Line"**

2

3 The present invention relates to the production of
4 cell lines which secrete insulin. More specifically
5 the invention relates to insulin producing cell
6 lines derived from human pancreatic islet cells.

7

8 The limited supply of human islets and labour-intensive
9 methods for their isolation together with marked
10 functional variability are inherent problems, which
11 greatly restrict the number of biochemical and islet
12 transplantation studies being performed. Generation of
13 human pancreatic B-cell lines would provide, for a
14 first time, a practically unlimited supply of pure
15 insulin-secreting cells which can be grown and
16 harvested with a minimum of effort. Such human cell
17 lines would be extremely useful in studies of
18 pancreatic B-cell biology. Further, such human cell
19 lines would be attractive alternatives to the use of
20 primary tissue in cell transplantation therapies for
21 type 1 diabetes.

22

1 A large number of rodent insulinoma cell lines have
2 been developed. Most have been derived from
3 transgenic animals expressing the SV-40 antigen
4 oncogene from the insulinoma gene promoter (Efrat *et*
5 *al.*, 1988; Hanahan, 1985; Santere *et al.*, 1981). Most of
6 the cell lines produced by these methods show inherent
7 defects including instability and failure to respond to
8 physiological stimulators of insulin secretion (Efrat
9 *et al.*, 1996). However, recent studies have succeeded
10 in duplicating many of the properties of fully
11 differentiated pancreatic B-cells (Knaack *et al.*,
12 1994). For example, a murine cell line from a
13 transgenic mouse in which an insulin promoter SV-40
14 antigen was inserted under the control of the
15 tetracycline-inducible system, was shown to reverse
16 hyperglycemia in a mouse diabetes model (Efrat *et al.*,
17 1995). These experiments clearly demonstrated the
18 potential of pancreatic B-cell lines for cell
19 transplantation and gene therapy of diabetes (Efrat,
20 1998).

21
22 Human insulin-producing cell lines would be a much more
23 desirable model, as opposed to rodent cell lines, for a
24 number of reasons. For clinical transplantation, a
25 major advantage of human cells over those from other
26 species is that there would be no need to overcome the
27 xenotransplantation barrier (Cooper *et al.*, 1994).
28 Allografts are much easier to protect from immune
29 rejection compared with xenografts. Hyperacute
30 rejection of xenografts is caused by recognition of
31 cell surface carbohydrate antigens by naturally
32 occurring antibodies (Cooper *et al.*, 1994). Xenografts

1 have raised also numerous ethical and safety concerns,
2 including the introduction of unknown animal pathogens
3 into the human population. Another concern is that
4 immunogenic peptides shed by xenografts may elicit
5 immune responses, which could result in cross-
6 reactivity against host proteins, thus potentially
7 triggering new autoimmune responses (Efrat, 1998).
8 Another advantage of human pancreatic B-cells is that
9 they are likely to be more compatible with the human
10 physiological environment compared with most animal B-
11 cells. Moreover, human endocrine cells differ from
12 other species in some of their biological properties.
13 For example, hepatocyte growth factor (HGF) has been
14 shown to be a potent mitogen for human pancreatic B-
15 cell, while in the mouse it is relatively inactive
16 (Hayek et al., 1995; Otonkoski et al., 1994).
17
18 Despite the overwhelming impetus to produce human
19 insulin-secreting cell lines, it has proven to be
20 extremely difficult to establish such cell lines from
21 the human pancreatic endocrine lineage, compared with
22 the rodent (Efrat et al., 1996; Wang et al., 1997).
23 One human insulinoma cell line has been developed from
24 a spontaneous insulinoma, but it apparently grows
25 slowly and has lost many differentiated characteristics
26 (Gueli et al., 1987). Other investigators have
27 attempted to immortalise foetal or adult human
28 pancreatic B-cells using transfection with oncogenes
29 (Wang et al., 1997). However this approach has been
30 foiled by inability of such cells to continue to
31 express insulin and their restricted growth rates *in*
32 *vitro* (Wang et al., 1997).

1 It is an aim of the present invention to provide a
2 human pancreatic cell line which is capable of
3 secreting insulin.

4
5 According to the present invention there is provided
6 a human pancreatic cell line, produced by
7 electrofusion of normal human islet cells with
8 immortal human cell lines wherein the human
9 pancreatic cell-line is capable of secreting
10 insulin.

11
12 Typical cell lines capable of secreting insulin are
13 chosen from the group of cell lines consisting of
14 the cell-line deposited under Accession No 00112811
15 at the European Collection of Cell Cultures (ECACC),
16 CAMR, Salisbury, Wiltshire on 28 November 2000 and
17 the cell-lines deposited under Accession Nos PTA
18 3523, PTA 3524 and PTA 3525 at the American Type
19 Culture Collection, 10801 University Boulevard,
20 Manassas, Virginia 20100-2209, USA on 17 July 2001.

21
22 The invention also provides a process for the
23 production of human pancreatic cell lines capable of
24 secreting insulin, the process including the steps
25 of electrofusing a mixture of normal human islet
26 cells with cells from at least one immortal human
27 cell line and incubating the mixture to generate
28 hybrid cells which are capable of secreting insulin.

29
30 Preferably the human islet cells and immortal human
31 cells are mixed in an approximate 1:1 ratio.

32

1 Preferably electrofusion occurs in a helical
2 chamber.

3

4 Preferably the electrofusion step includes exposing
5 the cells to a first pulse phase of AC field, a
6 second pulse phase of DC field and a third pulse
7 phase of AC field.

8

9 The first pulse phase may be comprised of between 5
10 to 10V, preferably 7V, 2MHZ of AC field for between
11 20-40, preferably 30 seconds.

12

13 The second pulse phase typically comprises of 40-
14 80V, preferably 60V, triple pulses of DC field with
15 each of the triple pulses being 10-20, preferably 15
16 seconds in duration.

17

18 The third pulse phase typically comprises of 5-10V,
19 preferably 7V, 2MHZ of AC field for 20-40,
20 preferably 30 seconds.

21

22 The media in which the cells are incubated typically
23 comprises hypoxanthine, aminopterin and thymidine.

24

25 Typically, hypoxanthine is present in the incubating
26 media at a concentration of between 0.05 μ mol/l to
27 0.5 μ mol/l.

28

29 Typically, aminopterin is present in the incubating
30 media at a concentration of between 0.2-0.6 μ mol/l.

31

1 Typically, thymidine is present in the incubating
2 media at a concentration of between 10-20 $\mu\text{mol/l}$.

3
4 The incubation may be carried out in the presence of
5 at least one secretagogue chosen from the group
6 comprising glucose, glyceraldehyde, arginine,
7 leucine and alanine.

8
9 The incubation may be carried out in the presence of
10 at least one substance chosen from the group
11 comprising KCl, IBMX, thioglucose, tolbutamide,
12 diazoxide and verapamil.

13
14 A cell line produced by the process of the invention
15 may exhibit glucose transport characteristics as
16 efficient as normal pancreatic B cells.

17
18 A cell line produced by the process of the invention
19 may exhibit glucose phosphorylating activity
20 consistent with normal pancreatic cells.

21
22 The invention also relates to the use of insulin
23 producing cells produced by the process of the
24 invention to provide gene therapy for type 1
25 diabetes.

26
27 The invention also relates to the use of insulin
28 producing cells produced by the process in the
29 preparation of a medicament for the treatment of
30 diabetes.

31

1 The invention also relates to the use of a cell-line
2 according to the invention in the preparation of a
3 medicament for the treatment of diabetes.

4

5 The invention also relates to the use of a cell-line
6 according to the invention for the production of
7 insulin.

8

9 The invention also relates to cells or materials
10 derived from the cell-lines disclosed herein and to
11 uses of these.

12

13 According to the present invention there is also
14 provided a process for the generation of human
15 pancreatic cell lines which are capable of secreting
16 insulin, the process including the step of
17 electrofusing normal human islet cells with immortal
18 human cell lines to generate hybrid cells which are
19 capable of expressing attributes of normal pancreatic
20 B-cells.

21

22 The invention thus provides human pancreatic cell-lines
23 capable of secreting insulin produced by an
24 electrofusing process.

25

26 In the examples the cell-line referred to under
27 Accession numbers above are referred under internal
28 reference numbers. These are as follows:

29

30	Accession No	Internal Reference
31	00112811	1.1B4
32	PTA 3523	1.1E7

1 PTA 3524 1.2B4

2 PTA 3525 1.4E7

3

4 A number of embodiments of the present invention will
5 now be described by way of example only. With
6 reference to the accompanying figures in which:

7

8 Figure 1 shows the typical steps involved in
9 the production of human insulin-secreting
10 pancreatic B-cell lines by electrofusion.

11

12 Figure 2 represents the insulin released into
13 the medium in the final 24 hours by cell
14 hybrids (azaguanine resistant) resulting from
15 electrofusion of human islet cells with TRM-1
16 cells after 40 days culture, control values are
17 mean \pm sem (n=6), other values are mean of
18 duplicate determinations, fusions 1 and 2
19 utilised islet preparations 1 and 2,
20 respectively.

21

22 Figure 3 represents the insulin released into
23 the medium in the final 24 hours by cell
24 hybrids (azaguanine resistant) resulting from
25 electrofusion of human islet cells with PANC-1
26 cells after 25 days culture, cells in A were
27 selected on the basis on high insulin output
28 for cloning, cells in B, clones 1.1B4 and 1.1E7
29 were selected on the basis of insulin output
30 for further evaluation, control values are mean
31 \pm sem (n=6), other values are mean of duplicate

1 determinations, fusion utilised islet
2 preparation 1.

3
4 Figure 4 represents the insulin released into
5 the medium in the final 24 hours by cell
6 hybrids (azaguanine resistant) resulting from
7 electrofusion of human islet cells with PANC-1
8 cells after 30 days culture, cells in well B
9 were selected on the basis on high insulin
10 output for cloning, (B) clone 1.1E7 was selected
11 for further evaluation, control values are mean
12 \pm sem (n=6), other values are mean of duplicate
13 determinations, fusions utilised islet
14 preparation 3.

15
16 Figure 5 represents the insulin released into
17 the medium in the final 24 hours by cell
18 hybrids (azaguanine resistant) resulting from
19 electrofusion of human islet cells with Hup-T₃-
20 1 cells after 30 days culture, cells in well C
21 were selected on the basis on high insulin
22 output for cloning, (B) clones 1.2B4 was
23 selected for further evaluation, control values
24 are mean \pm sem (n=6), other values are mean of
25 duplicate determinations, fusion utilised islet
26 preparation 3.

27
28 Figure 6 shows, (A) the mophology of PANC-1
29 cell lines and (B) the morphology of human
30 islet-derived 1.1B4 cell lines, using phase
31 contrast microscopy (x200 magnification).

32

1 Figure 7 shows (A) the morphology of human-
2 islet derived 1.1E7 cell line and (B) 1.4E7
3 cell line, using phase contrast microscopy
4 (x200 magnification).

5
6 Figure 8 shows (A) the morphology of Hup-T₃
7 cell line and (B) 1.2B4 human islet-derived
8 cell line, using phase contrast microscopy
9 (x200 magnification).

10
11 Figure 9 represents the cellular insulin
12 content of human islet-derived cell lines at
13 different passages, values are mean \pm sem
14 (n=8), there were no significant differences in
15 insulin content between cell lines or
16 increasing passage number.

17
18 Figure 10 represents the glucose responsiveness
19 of human islet-derived 1.1B4 (A), 1.1E7 (B),
20 1.4E7 (C) and 1.2B4 (D) cells, following 40 min
21 of preincubation, effects of various glucose
22 concentrations were tested during 60 min
23 incubations, values are mean \pm sem (n=12), *p
24 <0.05, **p<0.01, ***p<0.001 compared with 0 mM
25 glucose.

26
27 Figure 11 represents the insulin released from
28 human islet-derived clones in 1.1 mM glucose as
29 a % of the cellular insulin content, following
30 40 min preincubation, effects of 1.1 mM glucose
31 were tested during 60 min incubation, values
32 are mean \pm sem (n=12).

1
2 Figure 12 represents the glucose responsiveness
3 of human islet-derived 1.1B4 (A), 1.1E7 (B)
4 1.4E7 (C) and 1.2B4 (D) cells in the presence
5 of IBMX (200 μ M), following 40 min
6 preincubation, effects of various glucose were
7 tested in the presence of IBMX (200 μ M) during
8 60 min incubations, values are mean \pm sem
9 (n=12), *p <0.05, **p<0.01, ***p<0.001 compared
10 with 0 mM glucose in presence of 200 μ M IBMX).
11

12 Figure 13 represents the effects of IBMX (200
13 μ M) on glucose responsiveness of human islet-
14 derived 1.1B4 cells, following 40 min
15 preincubation, insulin secretion was measured
16 after 60 min incubations in the presence of the
17 indicated secretagogues, values are mean \pm sem
18 (n=12), $\Delta\Delta\Delta$ p <0.001 compared with control at
19 the same glucose concentration.
20

21 Figure 14 represents the effects of IBMX (200
22 μ M) on glucose responsiveness of human islet-
23 derived 1.1E7 cells, following 40 min
24 preincubation, insulin secretion was measured
25 after 60 min incubations in the presence of the
26 indicated secretagogues, values are mean \pm sem
27 (n=12), $\Delta\Delta\Delta$ p <0.001 compared with control at
28 the same glucose concentration.
29

30 Figure 15 represents the effects of IBMX (200
31 μ M) on glucose responsiveness of human islet-

1 derived 1.4E7 cells, following 40 min
2 preincubation, insulin secretion was measured
3 after 60 min incubations in the presence of the
4 indicated secretagogues, values are mean \pm sem
5 (n=12), $\Delta p < 0.05$, $\Delta\Delta\Delta p < 0.001$ compared with
6 control at the same glucose concentration.

7
8 Figure 16 represents the effects of IBMX (200
9 μ M) on glucose responsiveness of human islet-
10 derived 1.2B4 cells, following 40 min of
11 preincubation, insulin secretion was measured
12 after 60 min incubations in the presence of the
13 indicated secretagogues, values are mean \pm sem
14 (n=12).

15
16 Figure 17 represents the glucose responsiveness
17 of human islet-derived 1.1B4 (A), 1.1E7 (B),
18 1.4E7 (C) and 1.2B4 cells in the presence of 5-
19 thioglucose (2 mM), following 40 min of
20 preincubation, effects of various glucose
21 concentrations were tested in the presence of
22 5-thioglucose (2 mM) during 60 min incubations,
23 values are mean \pm sem (n=12) *p < 0.05, **p
24 < 0.01, ***p < 0.001.

25
26 Figure 18 represents the effects of 5-
27 thioglucose (2 mM) on glucose responsiveness of
28 human islet-derived 1.1B4 cells, following 40
29 min preincubation, insulin secretion was
30 measured after 60 min incubations in the
31 presence of the indicated secretagogues, values

1 are mean \pm sem (n=12), $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$
2 compared with control at the same glucose
3 concentration.

4
5 Figure 19 represents the effects of 5-
6 thioglucose (2 mM) on glucose responsiveness of
7 human islet-derived 1.1E7 cells, following 40
8 min preincubation, insulin secretion was
9 measured after 60 min incubations in the
10 presence of the indicated secretagogues, values
11 are mean \pm sem (n=12), $\Delta\Delta\Delta p < 0.001$ compared
12 with control at the same glucose concentration.

13
14 Figure 20 represents the effects of 5-
15 thioglucose (2 mM) on glucose responsiveness of
16 human islet-derived 1.1E7 cells, following 40
17 min preincubation, insulin secretion was
18 measured after 60 min incubations in the
19 presence of the indicated secretagogues, values
20 are mean \pm sem (n=12), $\Delta p < 0.05$, $\Delta\Delta p < 0.01$
21 compared with control at the same glucose
22 concentration.

23
24 Figure 21 represents the effects of 5-
25 thioglucose (2 mM) on glucose responsiveness of
26 human islet-derived 1.2B4 cells, following 40
27 min preincubation, insulin secretion was
28 measured after 60 min incubations in the
29 presence of the indicated secretagogues, values
30 are mean \pm sem (n=12), $\Delta\Delta\Delta p < 0.001$ compared
31 with control at the same glucose concentration.

32

1 Figure 22 represents the effects of known
2 stimulators of pancreatic B-cell function on
3 insulin secretion from human islet-derived
4 1.1B4 cells at 5.6 or 11.1 mM glucose, graph A
5 and B respectively, following 40 min of
6 preincubation, effects of various additions at
7 5.6 (A) or 11.1 (B) mM glucose were tested,
8 values are mean \pm sem (n=12), *p<0.05,
9 **p<0.01, ***p <0.001 compared with 5.6 (A) or
10 11.1 (B) mM glucose alone.

11
12 Figure 23 represents the effects of known
13 stimulators of pancreatic B-cell function on
14 insulin secretion from human islet-derived
15 1.1E7 cells at 5.6 or 11.1 mM glucose, graph A
16 and B respectively, following 40 min of
17 preincubation, effects of various additions at
18 5.6 (A) or 11.1 (B) mM glucose were tested,
19 values are mean \pm sem (n=8), *p<0.05, **p<0.01,
20 ***p <0.001 compared with 5.6 (A) or 11.1 (B) mM
21 glucose alone.

22
23 Figure 24 represents the effects of known
24 stimulators of pancreatic B-cell function on
25 insulin secretion from human islet-derived
26 1.1E7 cells at 5.6 or 11.1 mM glucose, graph A
27 and B respectively, following 40 min of
28 preincubation, effects of various additions at
29 5.6 (A) or 11.1 (B) mM glucose were tested,
30 values are mean \pm sem (n=12), *p<0.05,

1 **p<0.01, ***p <0.001 compared with 5.6 (A) or
2 11.1 (B) mM glucose alone.

3
4 Figure 25 represents the effects of known
5 stimulators of pancreatic B-cell function on
6 insulin secretion from human islet-derived
7 1.2B4 cells at 5.6 or 11.1 mM glucose, graph A
8 and B respectively, following 40 min of
9 preincubation, effects of various additions at
10 5.6 (A) or 11.1 (B) mM glucose were tested,
11 values are mean \pm sem (n=12), *p<0.05,
12 **p<0.01, ***p <0.001 compared with 5.6 (A) or
13 11.1 (B) mM glucose alone.

14
15 Figure 26 represents the effects of known
16 stimulators of pancreatic B-cell function on
17 insulin secretion from human islet-derived
18 1.1B4, 1.1E7, 1.4E7 and 1.2B4 cells, graph A,
19 B, C and D respectively, following 40 min of
20 preincubation, effects of various additions at
21 16.7 mM glucose during 60 min incubations,
22 values are mean \pm sem (n=12), *p<0.05,
23 **p<0.01, ***p <0.001 compared with 16.7 mM
24 glucose alone, effects of EGTA were determined
25 in calcium free buffer.

26
27 Figure 27 represents the effects of passage
28 number on stimulating insulin secretion from
29 human islet-derived insulin secreting cell
30 lines, following 40 min of preincubation,
31 effects of various additions were tested during

1 a 60 min incubation period, values are mean \pm
2 sem (n=12), *p<0.05, **p<0.01, ***p <0.001
3 compared with 0 mM glucose, there were no
4 significant differences between passage 17 and
5 40.

6
7 Figure 28 shows a western blotting analysis for
8 GLUT-1 glucose transporter protein in (A) PANC-
9 1 and derived clonal human islet cells and (B)
10 Hup-T₃ and derived clonal human islet cells,
11 where L is liver from Wistar rat which was used
12 as a positive control and C represents parental
13 cells of PANC-1 and Hup-T₃ respectively.

14
15 Figure 29 shows a western blotting analysis for
16 glucokinase protein in (A) PANC-1 and derived
17 clonal human islet cells and (B) Hup-T₃ and
18 derived clonal human islet cells, where L is
19 liver from Wistar rat which was used as a
20 positive control and C represents parental
21 cells of PANC-1 and Hup-T₃ respectively.

22
23 Figure 30 illustrates 3-O-methyl-D-[1-
24 ³H]glucose uptake by PANC-1 cells or human
25 islet-derived 1.1B4 cells at low and high
26 glucose concentration, incubations were
27 performed at 37°C at 1.1 or 16.7 mM glucose for
28 periods of 0 to 360 seconds. Values are mean \pm
29 sem (n=3), ***p <0.001 when compared with PANC-
30 1 at 16.7 mM glucose, Δ p <0.05 when compared
31 with PANC-1 at 1.1mM glucose.

1 Figure 31 illustrates 3-O-methyl-D-[1-
2 ³H]glucose uptake by PANC-1 cells or human
3 islet-derived 1.1E7 cells at low and high
4 glucose concentration, incubations were
5 performed at 37°C at 1.1 or 16.7 mM glucose for
6 periods of 0 to 360 seconds, values are mean ±
7 sem (n=3), ***p <0.001 when compared with PANC-
8 1 at 16.7 mM glucose.

9
10 Figure 32 illustrates 3-O-methyl-D-[1-
11 ³H]glucose uptake by PANC-1 cells or human
12 islet-derived 1.1E7 cells at low and high
13 glucose concentration, incubations were
14 performed at 37°C at 1.1 or 16.7 mM glucose for
15 periods of 0 to 360 seconds, values are mean ±
16 sem (n=3), *p <0.05, ***p <0.001 when compared
17 with PANC-1 at 16.7 mM glucose.

18
19 Figure 33 illustrates 3-O-methyl-D-[1-
20 ³H]glucose uptake by Hup-T₃ cells or human
21 islet-derived 1.2B4 cells at low and high
22 glucose concentration, incubations were
23 performed at 37°C at 1.1 or 16.7 mM glucose for
24 periods of 0 to 360 seconds, values are mean ±
25 sem (n=3), *p <0.01, ***p <0.001 when compared
26 with Hup-T₃ at 16.7 mM glucose.

27
28 Figure 34 illustrates glucose phosphorylating
29 activities of PANC-1 and human islet-derived
30 1.1B4, 1.1E7 and 1.4E7 cells, soluble
31 cytoplasmic fractions were assayed

1 spectrophotometrically to determine the
2 relative contributions of glucokinase and
3 hexokinase to the total glucose phosphorylating
4 activities of the cells, values are mean \pm sem
5 (n=3), *p < 0.01, ***p < 0.001 compared with
6 PANC-1 cells, the percentage contribution to
7 the total glucose phosphorylating activity is
8 given in parentheses.

9
10 Figure 35 illustrates glucose phosphorylating
11 activities of Hup-T₃ and human islet-derived
12 1.2B4 cells, soluble cytoplasmic fractions were
13 assayed spectrophotometrically to determine the
14 relative contributions of glucokinase and
15 hexokinase to the total glucose phosphorylating
16 activities of the cells, values are mean \pm sem
17 (n=3), there were no significant differences
18 between the two cell types, the percentage
19 contribution to the total glucose
20 phosphorylating activity is given in
21 parentheses.

22
23 Figure 36 illustrates glucose oxidation from D-
24 [U-¹⁴C]glucose in PANC-1 and human islet-derived
25 1.1B4, 1.1E7 and 1.4E7 cells, incubations were
26 performed at 37°C at various glucose
27 concentrations, values are mean \pm sem (n=3),
28 *p < 0.05, **p < 0.01, ***p < 0.001 when compared
29 with 1.1 mM glucose.

30

1 Figure 37 illustrates the effects of 5-
2 thiogluco^{se} on glucose oxidation from D- [U-¹⁴C]
3 glucose in PANC-1, human islet-derived 1.1B4,
4 1.1E7 and 1.4E7 cells, incubations were
5 performed at 37°C at various glucose
6 concentrations, values are mean ± sem (n=3),
7 ΔΔΔp <0.001 when compared with same glucose
8 concentration in absence of 5-thiogluco^{se},
9 *p<0.05, **p<0.01, ***p<0.001 when compared
10 with 1.1 mM glucose in the absence of 5-
11 thiogluco^{se}.

12
13 Figure 38 illustrates glucose utilization from
14 D- [5-³H] glucose in PANC-1, and human islet-
15 derived 1.1B4, 1.1E7 and 1.4E7 cells in the
16 presence or absence of 5-thiogluco^{se},
17 incubations were performed at 37°C at various
18 glucose concentrations, values are mean ± sem
19 (n=3), ***p<0.001 when compared with 1.1 mM
20 glucose in the absence of 5-thiogluco^{se}, Δp
21 <0.05 compared with the same glucose
22 concentration in the absence of 5-thiogluco^{se}.

23
24 Figure 39 illustrates glucose oxidation from D-
25 [U-¹⁴C]glucose in Hup-T₃ and human islet-derived
26 1.2B4 cells, incubations were performed at 37°C
27 at various glucose concentrations, values are
28 mean ± sem (n=3), *p<0.05, **p<0.01, ***p<0.001
29 when compared with 1.1 mM glucose.

30

1 Figure 40 illustrates the effects of 5-
2 thiogluco^{se} on glucose oxidation from D- [U-¹⁴C]
3 glucose in Hup-T₃ and human islet-derived 1.2B4
4 cells, incubations were performed at 37°C at
5 various glucose concentrations, values are mean
6 ± sem (n=3), $\Delta\Delta p < 0.01$, $\Delta\Delta\Delta p < 0.001$ when
7 compared with the same glucose concentration in
8 the absence of 5-thiogluco^{se}, $**p < 0.01$,
9 $***p < 0.001$ when compared with 1.1 mM glucose in
10 the absence of 5-thiogluco^{se}.

11
12 Figure 41 illustrates glucose utilization from
13 D- [5-³H] glucose in Hup-T₃ and human islet-
14 derived 1.2B4 cells in the presence or absence
15 of 5-thiogluco^{se}, incubations were performed at
16 37°C at various glucose concentrations, values
17 are mean ± sem (n=3), $***p < 0.001$ when compared
18 with 1.1 mM glucose in the absence of 5-
19 thiogluco^{se}, $\Delta\Delta\Delta p < 0.001$ when compared with the
20 same glucose concentration in the absence of 5-
21 thiogluco^{se}.

22
23 Figure 42 shows immunocytochemical staining for
24 cellular glucokinase (x400 magnification).

25
26 Figure 43 shows immunocytochemical staining for
27 cellular insulin (x400 magnification).

28
29 Figure 44 shows immunocytochemical staining for
30 cellular islet amyloid polypeptide (IAPP) (x400
31 magnification).

1 Figure 45 shows immunocytochemical staining for
2 cellular glucagon (x400 magnification).

3

4 Figure 46 shows immunocytochemical staining for
5 cellular somatostatin (x400 magnification).

6

7 Figure 47 - Table 1 : Details regarding human
8 islet preparations.

9

10 Figure 48 - Table 2 : 3-0-Methyl-D- [1-³H]
11 glucose uptake kinetics of PANC-1, HuP-T3 and
12 human islet-derived insulin-secreting cell
13 lines.

14

15 Figure 49 - Table 3 : Half maximal
16 equilibration time of 3-0-methyl-D- [1-³H]
17 glucose uptake of kinetics in PANC-1, HuP-T3
18 and human islet-derived insulin-secreting cell
19 lines.

20

21 Figure 50 - Table 4 : Relative metabolic flux
22 through oxidative glucose metabolism in HuP-T3,
23 PANC-1 and human islet-derived insulin-
24 secreting cell lines incubated in 1.1 or 16.7
25 mmol/l glucose in the absence and presence of
26 5-thioglucoase (2 mmol/l).

27

28 Figure 51 - Table 5 : Summary of
29 immunocytochemical investigation of functional
30 proteins in parental and novel human islet cell
31 lines.

32

1 Figure 52 - Table 6 : Summary of functional
2 characteristics of parental and novel human
3 islet cell lines.

4
5 Figure 53 - Table 7 : Identity profile results
6 of 1.4E7.

7
8 Figure 54 - Table 8 : Identity profile results
9 of 1.1E7.

10
11 Figure 55 - Table 9 : Identity profile results
12 of 1.1B4.

13
14 Figure 56 - Table 10 : Identity profile results
15 of 1.2B4.

16
17 The following embodiments of the present invention
18 describe the use of optimised electrofusion parameters
19 for the generation of novel human insulin-secreting
20 cell lines. Detailed studies are reported on the
21 characteristics and functional properties of the
22 established human pancreatic B-cell clones.
23 Human islets were isolated by Prof. R. Gomis
24 (Barcelona, Spain) and sent in tissue culture medium by
25 overnight courier to Coleraine. Three donor pancreas
26 were employed under local ethical approval. The first
27 batch of 400 islets from a 25 year old male donor were
28 received on 13/6/1997. The second batch of 400 islets
29 from a 30 year old male donor were received on
30 17/10/1997. The third batch of 300 islets from a 28
31 year old male were received on 3/12/1997 (Table 1).
32 Islets were disaggregated to single cells and cultured

1 overnight in RPMI 1640 culture medium prior to
2 electrofusion studies.

3

4 Immortal PANC-1 and Hup-T₃ cell lines were obtained
5 from ACECC at passage 60 and 52, respectively. PANC-1
6 is an epithelial cell line derived from a pancreatic
7 carcinoma of ductal origin from a 56 year old Caucasian
8 male (Lieber et al., 1975). Hup-T₃ cell line is an
9 epithelial cell line derived from ascites taken from a
10 66 year old Japanese male (Nishimura et al., 1993). The
11 immortal islet-derived human cell fusion partners, TRM-
12 1, HAP5 and B6 were provided by Prof. Hayek
13 (California, USA) and have a passage number of 8, 15
14 and 10, respectively.

15

16 Viability of cells prepared from the three preparations
17 of human islet cells, assessed by trypan blue exclusion
18 after overnight culture, was 67%, 39% and 71%,
19 respectively (Table 1). The islet cells were then
20 resuspended in fusion medium at a density of 2×10^6
21 cells/ml. The human pancreatic cell lines (PANC-1,
22 Hup-T₃, TRM-1, B6 or HAP6) were harvested, washed twice
23 with fusion medium and resuspended in fusion medium at
24 a density of 2×10^6 cells/ml. Both types of cells
25 were mixed at ratio of 1:1 and 300 μ l of cell mixture
26 were pipetted into helical chamber (Biojet, B.Braun
27 Biotech., Germany). This helical chamber was designed
28 closely identical with frame chamber but operated in
29 sterile conditions. The helical chamber was connected
30 with power supply and then exposed in 7 V, 2 MHz of
31 AC field for 30 seconds followed by 60 V, triple pulses
32 of 15 seconds duration times of DC field. The cells

1 were then exposed in 7V, 2 MHz of AC field for 30
2 seconds as post-fusion alignment.

3
4 The fusion mixture was then flushed from the helical
5 chamber with RPMI 1640 culture medium and equally
6 divided into a 24 well plate. Cells were incubated
7 overnight at 37°C. RPMI 1640 culture medium
8 supplemented with HAT (0.1 mmol/l hypoxanthine, 0.4
9 μmol/l aminopterin and 16.0 μmol/l thymidine) was
10 replaced after 24 hours culture and the cells were
11 maintained in this selective medium for 10 days period.
12 The cell fusion mixture was then cultured in RPMI 1640
13 culture medium supplemented with HT (0.1 mmol/l
14 hypoxanthine and 16.0 μmol/l thymidine) and cells were
15 maintained in this medium until colonies of hybrids
16 were seen under microscopic observation.

17
18 Colonies of hybrid cells normally appeared after 10 to
19 20 days of fusion as observed under an inverted
20 microscope. Colonies were maintained for another 10-20
21 days before medium was replaced for 24 hours before
22 screening. A single screening procedure was used to
23 select hybrid insulin-secreting cells from fusion
24 mixture. In order to determine accurately the insulin
25 concentration in the samples, controls were prepared
26 using culture media alone or media from 24 hours
27 culture of parental cells. This approach allowed
28 automatic correction for the possible presence of
29 insulin or interfering substances in the culture
30 medium.

31

1 Aliquots of 200 μ l culture medium were removed in
2 duplicate and the insulin concentration released into
3 culture medium was measured by radioimmunoassay as
4 described below. Wells containing hybrid insulin-
5 secreting cells were selected and cells from each well
6 were harvested and divided into 24 well plate for the
7 next screening step.

8

9 The novel human insulin-secreting cell lines were
10 cultured in RPMI 1640 culture medium supplemented with
11 10% (v/v) foetal bovine serum and antibiotics (100 μ g/ml
12 penicillin and 0.1 g/l streptomycin). The cells were
13 maintained under culture condition (37°C, 5% CO₂ and 95%
14 air) before the cells were used for experiments. Where
15 appropriate cells were cryopreserved and stored.

16

17 Cells were harvested with the aid of trypsin and then
18 resuspended in tissue culture medium. Cells were
19 seeded at a density of 5×10^5 cells per well in 6 well
20 plate and allowed to attach overnight in the 37°C
21 incubator. Following the removal of tissue culture
22 medium, the growing cell monolayers were then viewed
23 using a phase contrast microscope (Zeiss, Germany) and
24 images were captured by Imagedok version 2.0 software
25 package (Kinetic Imaging Ltd, UK).

26

27 Acute static incubations at 37°C were performed using
28 monolayers of insulin-secreting cells. Twenty four
29 hours prior to experimentation, the cells were
30 harvested and then resuspended in RPMI-1640 tissue
31 culture medium. Approximately 2.5×10^5 cells were then

1 seeded in each well of 24-well multiplates (Iwaki
2 Glass, Japan). After culture at 37°C and attachment of
3 cells overnight, the culture medium was removed and 1ml
4 of KRBB buffer (pH 7.4) supplemented with 1% (w/v)
5 bovine serum albumin (BSA) and 1.1 mmol/l glucose, was
6 carefully added to each well. The cells were incubated
7 at 37°C for 40 minutes, after which the buffer was
8 removed and replaced with 0.5 ml KRBB test buffer. The
9 KRBB test buffer was supplemented with 0.5 g/l (w/v)
10 bovine serum albumin (BSA) and 1.1 mmol/l glucose along
11 with the established modulators of pancreatic B-cell
12 function. After 60 min incubation at 37°C, a 450 µl
13 aliquot of KRBB test buffer was removed from each well
14 into 2ml polypropylene tube (Lip Plastics, England) and
15 centrifuged at 900 rpm for 5 minutes at 4°C. The
16 supernatant was then collected from each tube and 200µl
17 aliquots were then stored at -20°C for subsequent
18 insulin determination by radioimmunoassay.

19
20 After harvesting, the cells were resuspended in tissue
21 culture medium, seeded at a density of 2.5×10^5 cells
22 per well, and allowed to attach overnight to form
23 monolayers in 24 well multiplates (Iwaki Glass, Japan).
24 The culture medium was then completely removed and 500
25 µl of acid-ethanol solution (1.5% (v/v) HCl, 75% (v/v)
26 ethanol, 23.5% (v/v) H₂O) was added. The cells were
27 disrupted with the aid of a pasteur pipette and
28 incubated overnight at 4°C prior to centrifugation at
29 900 rpm for 5 minute. Supernatants were stored at -
30 20°C for subsequent determination of cellular insulin
31 content by radioimmunoassay.

1 Insulin was measured by modified dextran-charcoal
2 radioimmunoassay (Albano et al., 1972) as described by
3 Flatt and Bailey (1981). Working radioimmunoassay
4 phosphate buffer (40 mmol/l sodium phosphate buffer, pH
5 7.4 supplemented with 0.5% (w/v) bovine serum albumin)
6 was used to dilute human insulin standards, insulin
7 antibody, ^{125}I -labelled insulin and samples as
8 appropriate. Human insulin standards were prepared over
9 a range of 2-fold serial dilutions from 5.0 ng/ml to
10 0.009 ng/ml using Human insulin standard (Sigma
11 Chemical, UK). 200 μl of the standard (pipetted in
12 triplicate) or unknown sample (pipetted in duplicate)
13 was mixed with 100 μl of guinea pig anti-bovine insulin
14 antiserum at around 1: 100,000 dilution) and incubated
15 for 24 hours at 4°C prior to the addition of 100 μl of
16 ^{125}I -insulin (approx. 100 pg.). This antiserum was
17 specifically selected for high affinity with human
18 insulin standard.

19

20 After a further 24 hours incubation at 4°C, 1ml of a 5%
21 (w/v) charcoal coated with 1:10 dextran T-70 in sodium
22 phosphate buffer (40 mmol/l, pH 7.4) was added to each
23 tube. After 20 minutes incubation (4°C), each tube was
24 centrifuged at 2500 rpm for 20 minutes (4°C). The
25 supernatant was decanted and the counts of ^{125}I -insulin
26 bound to charcoal were recorded using a gamma counter
27 (1261 Multigamma Counter, LKB Wallac, Turku, Finland)
28 linked to a computer (Olivetti PCS 286). Counts bound
29 to antibody (average total counts minus counts bound to
30 charcoal) are inversely proportional to the
31 concentration of insulin in the standard or unknown

1 sample. The unknown concentration was determined by
2 means of the standard curve, constructed from the known
3 rat insulin standard using a spline curve fitting
4 algorithm. This assay gives 10%, 50% and 90% fall in
5 bond counts an addition of 19 pg/ml, 250 pg/ml and 2.5
6 ng/ml human insulin, respectively.

7
8 Protein concentration were determined using a
9 modification of the method described by Bradford
10 (1979). The Bio-Rad protein assay is a dye binding
11 assay based on the differential absorbance change of
12 Coomassie Brilliant Blue G250 under acidic conditions
13 in response to various concentrations of protein. The
14 Bio-Rad protein assay reagent was diluted with
15 distilled water and glacial acetic acid in the ratio
16 4:5:1 respectively. This was then filtered through
17 Whatman No. 1 grade filter prior to use. Known
18 standards were prepared in the range of 1-20 $\mu\text{g}/\mu\text{l}$ of
19 bovine serum albumin in distilled water. The reagent
20 (200 μl) and 25 μl sample (standard protein or unknown)
21 were then mixed several times times by gentle inversion
22 in microtiter plate. After a period of 30 minutes, the
23 absorbance at 595 nm was measured on a microtiter plate
24 reader (Thermo, UK). A standard curve was produced
25 from the BSA standard each time the assay was
26 performed, and the sample protein concentration
27 determined.

28

29 The cell lines (parental and clonal) were harvested
30 (approximately 5×10^7 cell per preparation) and
31 resuspended in phosphate buffer saline, pH 7.4.
32 Following 6 cycles of sonication (10 seconds/cycle)

1 using a Soniprep 150 (MSE, UK), the cell lysates were
2 centrifuged for 10 minutes at 10,000 rpm (Beckman
3 Ultracentrifuge, USA). The supernatant was collected
4 and centrifuged for another 1 hour at 45,000 rpm. The
5 membrane pellets obtained were resuspended in PBS and
6 protein membrane concentration was measured by the Bio-
7 Rad assay as described above.

8
9 Insulin-secreting cells were harvested (approximately 4
10 $\times 10^7$ cells per preparation) and resuspended in ice-
11 cold sonication medium (20 mmol/l Hepes, 210 mmol/l
12 mannitol, 70 mmol/l sucrose, adjusted to pH 7.4 using
13 KOH, supplemented with 1 mmol/l dithiotreitol and 5%
14 glycerol). The cells were then sonicated (Soniprep
15 150, MSE, UK) for 30 seconds at 4°C. A soluble
16 cytoplasmic fraction was obtained by three successive
17 centrifugation steps at 4°C of 5000 rpm, 10,000 rpm,
18 and 45,000 rpm, respectively (Lenzen et al., 1985).
19 Glucokinase protein concentration was measured by the
20 Bio-Rad assay as described above. Glucose
21 phosphorylating enzyme activity was determined using
22 the Varian Cary 1 UV/VIS spectrophotometer (Philips,
23 UK)), recording the formation of NADPH.
24 Rates of glucose phosphorylation in the 45,000 rpm
25 soluble cytoplasmic fractions were assayed at 37°C (pH
26 7.4) by recording the increase in absorbance at 340 nm
27 in 500 μ l mixture containing 20 mmol/l Hepes (adjusted
28 to pH 7.4 with KOH), 125 mmol/l KCl, 7.5 mmol/l $MgCl_2$,
29 5 mmol/l ATP, 0.5 mmol/l NADP, 700 U/I Glucose-6-
30 phosphate dehydrogenase, 10 U/I 6-P-gluconate
31 dehydrogenase plus cytoplasmic supernatant (50 μ l) from
32 each aliquot of cells. Hexokinase activity was assayed

1 in the presence of 1 mmol/l D-glucose and subtracted
2 from the total activity recorded at 100 mmol/l D-
3 glucose to give glucokinase activity (Lenzen *et al.*,
4 1985). One unit of enzyme activity was defined as 1
5 μ mole of glucose-6-phosphate formed from glucose and
6 ATP per minute at 37°C. Protein determination was
7 performed on the cytoplasmic supernatant using the Bio-
8 Rad assay, as previously described.

9
10 Insulin-secreting cells were seeded at a concentration
11 of 2×10^5 cells/well in 35 mm² tissue culture dishes
12 (Nuncclon, Denmark) and cultured for 24 hours at 37°C.
13 Cells were given two rapid washes in phosphate buffer
14 saline and then preincubated for 30 minutes at 37°C in
15 KRBB supplemented with 1.1 mmol/l glucose and 0.1% BSA.
16 Cells were then incubated with 0.5ml of KRBB
17 supplemented with 3-O-methyl-D-[1-³H] glucose (Amersham
18 Pharmacia, USA) for 0, 5, 10, 20, 60, 120, 240 and 360
19 seconds in 37°C. Tested concentrations of 1 μ Ci/ml 3-
20 O-methyl-D-[1-³H] glucose were used in KRBB containing
21 1.1 and 16.7 mmol/l D-glucose. During the incubation,
22 plates were stood on a hot plate heated to 37°C.
23 Uptake of tritiated with 3-O-methyl-D-[1-³H] glucose
24 was terminated with 3 rapid washes using 0.5ml ice-cold
25 KRBB containing 100 mmol/l glucose. Cells were then
26 lysed with 1ml 0.5% sodium dodecyl sulphate (SDS) and
27 left at room temperature for 20 minutes. Scintillation
28 liquid (HiSafe, BDH) was added (3ml) to the lysate and
29 mixed well. The vials were left to stand for 3 hours
30 in the dark to allow any bubbles to rise to the
31 surface. The counts of radioactivity in the lysate was

1 quantified by liquid scintillation counting (Wallac
2 1409 liquid scintillation counter, Finland).
3
4 A modification of the method developed by Keen *et al.*
5 (1963) was used to determine oxidative glucose
6 metabolism to CO₂. In principle, the method involves
7 incubation of cells in specially designed glass cups
8 suspended inside standard counting vials with an
9 airtight rubber stopper. A disc of filter paper
10 (Whatman No. 1) was placed on the base of the counting
11 vial. For each sample, 2.0 x 10⁵ cells were incubated
12 in the presence of either 1.1, 5.6, 11.1, 16.7 mmol/l
13 glucose and D-[U-¹⁴C] glucose to give a final
14 concentration of 0.25µCi (40µl total volume). Effect
15 of 2 mmol/l 5-thioglucoase were also tested in 1.1 and
16 16.7 mmol/l glucose. Blanks were set up where the cells
17 were omitted and replaced with KRBB to give a final
18 concentration of either 1.1 or 16.7 mmol/l glucose.
19 Standards consisted of ¹⁴C-labelled glucose (0.5µCi)
20 added to the filter paper at the base of the counting
21 vial. The glass vials were sealed with the rubber caps
22 and placed at 37°C for one hour. The reaction was
23 stopped by the addition of 0.2M HCl (50µl) injected
24 through the rubber cap directly into the centre well
25 using a Hamilton syringe. Phenylethylamine (PEA) was
26 added (100µl) through the sealed lid onto the filter
27 paper at the base of the vial. Care was taken to avoid
28 contamination of the centre well with PEA. Vials were
29 then placed at 37°C for one hour. The rubber caps and
30 centre wells were removed and 5ml of liquid
31 scintillation fluid (BDH) added to each vial on top of

1 the filter paper. Vials were capped and ^{14}C - glucose
2 labelled radioactivity determined by scintillation
3 counter.

4
5 Experiments were performed in specially designed glass
6 cups suspended inside standard counting vials with an
7 airtight stopper. For each sample, 2.0×10^5 cells were
8 incubated in the presence of either 1.1, 5.6, 11.1 or
9 16.7 mmol/l glucose and D-[5- ^3H] glucose to give a
10 final concentration of 0.25 μCi (40 μl total volume).
11 Effects of 2 mmol/l 5-thioglucoase were also tested in
12 1.1 and 16.7 mmol/l glucose. Blanks were set up where
13 the cells were omitted and replaced with KRBB to give a
14 final concentration of either 1.1 or 16.7 mmol/l
15 glucose. Standards consisted of D-[5- ^3H] glucose
16 (0.5 μCi) added to the base of the counting vial. The
17 glass vials were sealed with the rubber caps and placed
18 at 37°C for one hour. The reaction was stopped by the
19 addition of 0.2M HCl (50 μl) injected through the rubber
20 cap directly into the centre well using a Hamilton
21 syringe. Sterile deionized water was added (500 μl)
22 through the sealed lid into the base of the vial.
23 Vials were then placed at 37°C for a 15 hour. The
24 rubber caps and centre wells were removed and 5ml of
25 liquid scintillation fluid (BDH) added to each vial on
26 top of the filter paper. Vials were capped and ^3H -
27 glucose labelled radioactivity determined by
28 scintillation counter.

29

30

31

1 SDS-PAGE Gel Preparation:

2

3 The following reagents and buffer were prepared:

4

5 Lower Gel Buffer: 1.5mmol/l Tris-HCl, pH 8.8
6 Upper Gel Buffer: 0.5 mmol/l Tris-HCl, pH 6.8
7 Electrode Buffer: 25 mmol/l Tris, 192 mmol/l
8 Glycine and 0.1% (w/v) SDS
9 Sample Buffer: 125 mmol/l Tris-HCl, pH 6.8
10 containing 4.6% (w/v)
11 sodium dodecyl sulphate (SDS),
12 30% (v/v) glycerol, 0.002% (w/v)
13 bromophenol blue and 10% (v/v)
14 2 β -mercaptoethanol.
15 Acrylamide: 30% (w/v) acrylamide.

16

17 A vertical slab mini-gel apparatus (Bio-Rad) was used
18 for this analysis. The gel was cast in a glass
19 cassette, with perspex slide held together in the gel
20 apparatus. The dimensions of the gel were 80mm x 73mm
21 x 1mm. The glass front and back were washed with decon
22 detergent, rinsed with tap water and then distilled
23 water and finally cleaned with acetone and left to air
24 dry. The cassette was assembled and clamped into a
25 vertical position.

26

27 The lower gel was prepared by mixing the following
28 reagents in a 25ml universal tube: lower gel buffer
29 (2.5ml), distilled water (4.2ml), SDS 10% (w/v)
30 (100 μ l), acrylamide (3.3ml) and ammonium persulfate 10%
31 (w/v) (50 μ l). Polymerisation was initiated by the
32 addition of 5 μ l of N,N,N',N' -tetramethyl-

1 ethylenediamine (TEMED). The solution was poured into
2 the cassette to a height of 10 cm. A thin layer of
3 distilled water was placed on top of the gel solution
4 to ensure a flat interface between the lower and upper
5 gel. Polymerisation of the lower gel was complete in
6 60-90 minutes, at which time the layer of water was
7 removed.

8
9 The upper stacking gel was then prepared by mixing the
10 following reagents in a 25ml universal tube: upper gel
11 buffer (1.25ml), distilled water (3.05ml), SDS 10%
12 (w/v) (50µl), acrylamide (670µl) and ammonium
13 persulfate 10% (w/v) (25µl). Polymerisation was
14 initiated by the addition of 5µl of N,N,N'N' -
15 tetramethyl-ethylenediamine TEMED and the upper
16 stacking gel poured into the cassette above the lower
17 gel. A perspex comb was introduced into the upper gel
18 to form the sample wells and after approximately 30
19 minutes the gel had polymerised. Protein content was
20 determined using the Bradford method as described
21 previously. The samples (GLUT-1 and glucokinase
22 protein preparations) were then boiled for 5 minutes in
23 a water bath to denature the protein prior to loading
24 into the sample wells. The comb was subsequently
25 removed forming sample wells into which protein sample
26 (containing 100µg protein) and protein biotinylated
27 molecular weight marker (Amersham, UK) were loaded.
28 The cassette was then placed into the electrophoresis
29 tank. The tank was then assembled and electrode buffer
30 was placed in the upper and lower reservoirs.
31

1 Electrophoresis was performed immediately following
2 sample loading at a constant current of 40mA, 120V
3 using a Pharmacia EPS500/400 power pack (Pharmacia, USA
4) until the bromophenol blue band had migrated to the
5 lower edge of the lower running gel, the current was
6 then switched off. The cassette was then removed from
7 the electrophoresis tank and the glass plates
8 separated, the lower gel was separated from the upper
9 stacking gel and used for the transfer of proteins onto
10 nitrocellulose for Western blotting analysis.

11

12 The following buffers were prepared for Western
13 blotting and detection. The transfer buffer comprised
14 Tris (20 mmol/l), glycine (150 mmol/l) and 15% (v/v)
15 of methanol. The wash buffer comprised phosphate
16 buffer saline (PBS) supplemented with 0.2% (v/v)
17 polyoxyethylene sorbitan monolaurate (Tween-20). The
18 blocking buffer comprised PBS supplemented with dried
19 skimmed milk 2.5% (w/v).

20

21 For Western blotting, the lower running gel was removed
22 from the cassette and placed in transfer buffer (30ml)
23 for 30 minutes to allow the buffer to permeate the gel
24 and replace the electrode buffer. During this time the
25 gel shrank as it equilibrated with the transfer buffer.
26 After equilibration, the gel was removed and placed
27 into the transfer cassette. The cassette was prepared
28 under transfer buffer and all the air bubbles removed
29 by rolling with a glass test tube.

30

31 Once assembled, the cassette was placed into a transfer
32 tank with the nitrocellulose sheet closest to the

1 positive electrode. The transfer was performed at a
2 constant current of 350mA, 100V for 90 minutes using
3 Bio-rad Transblot System (Richmond, CA, USA) according
4 to the method of Towbin et al. (1979). The cassette
5 was opened and the nitrocellulose membrane placed into
6 25ml blocking buffer and rocked for one hours followed
7 by incubation overnight at 4°C.

8
9 The nitrocellulose was washed in PBS-T solution five
10 times for five minutes each, followed incubation for 1
11 hour with the primary GLUT-1 antibody (Santa Cruz
12 Biotechnology, CA, USA) or glucokinase antibody (S.
13 Lenzen, Hanover, Germany) diluted (1:1000) in PBS. The
14 washing steps were repeated before a one hour
15 incubation of the membrane with horseradish peroxidase
16 labelled antibody (Amersham, UK) diluted (1: 1000) in
17 PBS. The membranes were then washed and incubated for
18 one hour in streptavidin- HRP (horse radish peroxidase)
19 conjugate diluted (1:1500) in PBS. After final washing
20 steps (consisting of 3 washes of 5 minutes duration in
21 wash buffer, followed by 2 washes in PBS alone) the
22 signal of protein expression was visualised using the
23 Amersham enhanced chemiluminescence (ECL) detection
24 system. Basically, this system works on the HRP/H₂O₂
25 catalysed oxidation of luminol in alkaline conditions,
26 in the presence of chemical enhancers such as phenols.
27 The light produced by this enhanced chemiluminescent
28 reaction can be detected by the short exposure (30
29 seconds) to blue-light sensitive autoradiography film,
30 Hyperfilm ECL (Amersham, UK).
31

1 Prior to immunohistochemical studies, each cell line
2 was plated onto 'supercell' incubation slides
3 containing 8 separate plastic chambers per slide
4 (BDH/Merck, UK.) and placed in culture at 37°C
5 overnight to allow the cells to adhere to the slides.
6 The plastic chambers holding the tissue culture medium
7 were carefully removed and the slides briefly washed in
8 phosphate buffered saline (PBS, Oxoid) before fixation
9 in ice-cold 4% paraformaldehyde (BDH/Merck)/PBS for 20
10 min. After washing with PBS, the cells were
11 permeabilised in a solution of 0.3% triton X-100 (Sigma
12 Chemicals) for 15 min. Further washes in PBS were
13 followed by blocking the cells with 2% normal goat
14 serum (Vector Laboratories, CA, USA)/1% BSA/PBS for 90
15 min at room temp. Incubation with the primary antibody
16 (insulin and glucagon guinea pig anti-porcine antisera
17 from Prof. PR. Flatt, used at a dilution of 1:1000;
18 Glut 1 affinity purified goat polyclonal antibody from
19 Santa Cruz biotechnology CA, USA, used at a dilution of
20 1;100; human somatostatin and amylin rabbit antisera
21 from Penninsula Laboratories Inc, Belmont, CA, USA, all
22 used at a dilution of 1:200; Glucokinase rat polyclonal
23 antisera, gift from Prof. Sigurd Lenzen (Hannover,
24 Germany), used at a dilution of 1:1000) was carried out
25 for 1 hour at 37°C, all dilutions were made in 0.2%
26 normal goat serum/0.1% BSA/PBS. After thorough washing
27 in PBS cells were incubated with fluorescein anti-
28 rabbit IgG (H+L) made in goat (Vector Laboratories, CA,
29 USA) at a dilution of 1:40 in PBS, for 45 min at 37°C.
30 Slides were given 5 x 5 min washes in PBS and mounted
31 under a glass coverslip in a solution of 50% glycerol
32 (Sigma Chemicals)/50% PBS. Immunohistochemical

1 staining was visualised using a Nikon OP-5 microscope
2 and images recorded using the PC *Imagedok* program.

3
4 The procedure for screening is summarized in Figure 1.
5 Hybrids cells produced by electrofusion were selected
6 through a screening procedure based on a high level of
7 insulin output. Basically, for one electrofusion
8 experiment the cell fusion mixture was divided into 24
9 wells and measurement of insulin output was taken over
10 the final 24 hours of the 30 to 35 day culture period.
11 Initial experiments were performed using the immortal
12 islet-derived human cell fusion partners, TRM-1, HAP5
13 and B6 provided by Professor Hayek (California, USA).
14 The results of 2 fusion experiments between HAT-
15 sensitive TRM-1 cells and human islet cells are
16 summarised in Figure 2. Post-fusion selection revealed
17 surviving cell hybrids in 24 wells, with 13 wells
18 containing hybrids secreting insulin at 40 days.
19 Attempts to clone the insulin-releasing hybrids were
20 thwarted by rapidly declining growth rate indicating
21 that the TRM-1 cell line was not fully immortalised.
22 Similar results were obtained using HAP5 and B6 cells
23 (data not shown), at least illustrating an expected
24 yield of about 10 human hybrid cells per fusion. Two
25 fusion with HAP5 cells yielded 12 and 11 hybrids of
26 which 4 and 6 produced insulin at 12 days. The two B6
27 cells fusions yielded 15 and 13 hybrids with 5 and 3
28 releasing insulin at 6 days.

29
30 In view of problem with the initially adopted fusion
31 partners (TRM-1, HAP5 and B6), attention was focussed
32 on use of human pancreatic adenocarcinoma cells, PANC-

1 1 and Hup-T₃, obtained from European Collection of
2 Animal Cell Culture (ECACC). HAT-sensitive clones and
3 electrofusion parameters for these cell lines were
4 established. The screening procedure which involved
5 fusion between PANC-1 and human islets cells revealed 5
6 positive hybrid colonies hybrids from the total of 25
7 hybrid colonies (Figure 1, 3A, 4A). Cells from well A
8 and well B were selected for further evaluation. After
9 a series of cloning steps, the clones 1.1B4, 1.1E7 and
10 1.4E7 were isolated (Figure 3B-4B). The other 16
11 insulin-releasing clones were cryopreserved but not
12 studied further.

13

14 Cell line 1.1E7 was produced by fusion of human islet
15 cells with an established cell line PANC-1, 1.1E7, P27,
16 07052001.

17

18 Cell line 1.4E7 was produced by fusion of human islet
19 cells with an established cell line PANC-1, 1.4E7 P29,
20 07112001.

21

22 Similar screening procedures were taken for fusion
23 between Hup-T₃ cell lines and human islet cells (Figure
24 1). The first screening after 30 days of fusion showed
25 13 wells from a total of 48 wells contained hybrid
26 colonies, 4 of which were insulin-positive hybrids
27 (Figure 5A). Hybrid cells from well C were selected
28 for a series of cloning steps, after which the insulin-
29 releasing clone 1.2B4 was isolated (Figure 5B). The
30 other 7 insulin-releasing clones were cryopreserved.

31

1 Cell line 1.2B4 was produced by fusion of human islet
2 cells with an established cell line Hup-T₃, 1.2B4, P27,
3 07102001.

4
5 As shown in Figure 6-8, parental PANC-1, Hup-T₃ and the
6 derived human islet cell clones grew as monolayers in
7 tissue culture. When confluent each cell line took on
8 a pavemental pattern as is routinely observed with
9 epithelioid cell lines. The human islet hybrid cell
10 lines showed consistent growth patterns with
11 approximate doubling times of 23 ± 1.7 hours for 1.1B4
12 cells, 23 ± 1.9 for 1.1E7 cells, 26 ± 1.5 hours for
13 1.4E7 cells and 36 ± 2.1 hours for 1.2B4 cells (n=4).
14 All of the islet-derived cell lines maintained their
15 gross morphological appearance and doubling times at
16 passage 40. The approximate doubling times on non-
17 fused PANC-1 and Hup-T₃ were 21 ± 1.6 and 30 ± 2.3
18 hours (n=4), respectively.

19
20 As shown in Figure 9, the cellular insulin content
21 (mean \pm sem, n = 12) of 1.1B4, 1.1E7, 1.4E7 and 1.2B4
22 cells at passage 17 was 0.394 ± 0.019 , 0.354 ± 0.021 ,
23 0.351 ± 0.023 and $0.363 \pm .017$ ng/10⁶ cells
24 respectively. At passage 40, there was no significant
25 change in the cellular insulin content of each cell
26 line. Insulin contents of 0.408 ± 0.024 , $0.314 \pm$
27 0.018 , 0.361 ± 0.02 and 0.306 ± 0.02 ng/10⁶ cells were
28 observed for 1.1B4, 1.1E7, 1.4E7 and 1.2B4 cells,
29 respectively. PANC-1 and Hup-T₃ cells did not contain
30 immunoreactive insulin.

31

1 The effects of glucose on insulin secretion from each
2 of the 4 novel human islet cell lines cells are shown
3 in Figure 10. Each cell line showed a different
4 pattern and magnitude of responsiveness to acute
5 glucose concentrations exposure. 1.1B4 cells showed a
6 stepwise 2.3-fold insulin-secretory response to
7 increasing glucose concentration over the range 0
8 mmol/l to 16.7 mmol/l glucose, with a threshold for
9 insulin release at 5.6 mmol/l glucose. Both 1.1E7 and
10 1.4E7 cells showed maximal 1.6 and 1.5-fold insulin-
11 secretory response to glucose at 11.1 mmol/l and 5.6
12 mmol/l glucose, respectively. In contrast Hup-T₃-
13 derived 1.2B4 cells, showed no significant insulin
14 response to glucose over the range from 0 mmol/l to
15 16.7 mmol/l glucose.

16

17 The insulin release from human islet-derived clones in
18 1.1 mmol/l glucose as a % of the cellular insulin
19 content was shown in Figure 11. Basal insulin
20 secretion from each of the human islet-derived clones
21 represented between 9% - 11% of the cellular insulin
22 contents.

23

24 Figure 12 shows insulin secretory response to glucose
25 from each of the four cell lines in the presence of
26 isobutylmethylxanthine (IBMX). Inclusion of 200 µmol/
27 IBMX in the test buffer with various glucose
28 concentrations from 0 mmol/l to 16.7 mmol/l greatly
29 enhanced the stimulatory effect of glucose on 1.1B4,
30 1.1E7 and 1.4E7 cells. As shown in Figure 13-15, the
31 secretory response was significantly increased by 20 to
32 100% to 1.3-2 fold stimulation ($p < 0.001$) of 1.1B4,

1 1.1E7 and 1.4E7 cells. However, inclusion of IBMX did
2 not significantly affect insulin released from 1.2B4
3 (Figure 16).

4
5 The effects of 5-thioglucoſe on the acute glucose
6 responsiveness of the human cell lines are shown in
7 Figure 17. Responsiveness of all four types of cell to
8 increasing of glucose concentrations was improved by
9 the inclusion of 5-thioglucoſe in the incubation
10 buffer. Although 5-thioglucoſe did not appear to
11 enhance the stimulating effect of glucose, it greatly
12 reduced basal insulin release and caused a generalised
13 right shift in the glucose responsiveness of 1.1B4,
14 1.1E7 and 1.2B4 cell lines toward more physiological
15 concentrations. Comparison of absolute rates of
16 insulin release indicated that inclusion of 5-
17 thioglucoſe significantly lowered insulin output from
18 1.1B4, 1.1E7 and 1.2B4 by 20 - 50% ($p < 0.05$ to $p < 0.001$;
19 Figures 18,19,21). Of the cell lines tested, 1.4E7
20 cells were least affected by inclusion of 5-thioglucoſe
21 in the incubations (Figure 19).

22
23 As shown in Figures 22-25, the effects of a range of
24 nutrient and pharmaceutical secretagogues were tested
25 at two different glucose concentrations (5.6 mmol/l and
26 11.1 mmol/l). Each cell line showed differences in
27 both the magnitude and pattern of responsiveness.

28
29 The secretory responses of 1.1B4 cells to a range of
30 modulators tested at 5.6 mmol/l glucose are shown in
31 Figure 22A. All stimulators, including glyceraldehyde,
32 leucine, KIC, arginine, alanine, tolbutamide and a

1 depolarising concentration of KCl, significantly
2 ($p < 0.05$ to $p < 0.001$) increased insulin secretion by 1.2-
3 2 fold. As shown in Figure 22B, increasing the glucose
4 concentration from 5.6 mmol/l to 11.1 mmol/l
5 significantly increased ($p < 0.01$ to $p < 0.001$) the effects
6 of leucine, arginine and tolbutamide by 30, 17 and 20%,
7 respectively.

8
9 The insulin secretory responses of 1.1E7 cells to the
10 same range of stimulators are shown in Figure 23. At
11 5.6 mmol/l glucose, glyceraldehyde, arginine and KCl
12 evoked significant 1.2-1.8 fold insulin secretory
13 responses ($p < 0.05$ to $p < 0.001$; Figure 23A). However
14 when tested at the higher glucose concentration of 11.1
15 mmol/l glucose, all stimulators elicited a significant
16 increase insulin output ($p < 0.05$ to $p < 0.001$; Figure
17 23B). Responses to leucine, KIC, alanine and arginine
18 were significantly greater at 11.1 mmol/l compared with
19 their effects at 5.6 mmol/l glucose ($p < 0.05$ to
20 $p < 0.001$).

21
22 The insulin secretory responses of 1.4E7 and 1.2B4
23 cells to a range of agents were similar (Figure 24 and
24 25). Thus at 5.6 mmol/l glucose, both cell lines
25 showed significant 1.2-1.7 fold insulin responses
26 ($p < 0.05$ to $p < 0.001$) when tested with glyceraldehyde,
27 arginine, tolbutamide or KCl. In the presence of 11.1
28 mmol/l glucose, the stimulatory actions of all agents
29 tested was significantly increased ($p < 0.05$ to $p < 0.001$;
30 Figure 24B and 25B). Furthermore leucine, KIC and
31 alanine each induced insulin secretion from 1.4E7 and

1 1.2B4 cells when tested at the higher glucose
2 concentration ($p < 0.05$ to $p < 0.01$).

3

4 The effects of the K^+ -ATP channel opener, diazoxide on
5 insulin release from 1.1B4, 1.1E7, 1.4E7 and 1.2B4
6 cells at 16.7 mmol/l glucose are shown in Figure 26.

7 Diazoxide (400 μ mol/l) caused a significant 20-50%
8 inhibition of insulin release from cell lines ($p < 0.01$
9 to $p < 0.001$).

10

11 Inclusion of the voltage-dependent Ca^{2+} channel (VDCC)
12 blocker, verapamil similarly inhibited insulin release
13 by 20-40% ($p < 0.05$ to $p < 0.001$) from each of the cell
14 lines (Figure 26). To evaluate the importance of
15 extracellular Ca^{2+} for glucose-induced insulin release,
16 a Ca^{2+} -free buffer supplemented with the Ca^{2+} -chelator,
17 EGTA was employed. As shown in Figure 26 depletion of
18 Ca^{2+} significantly decreased insulin secretion by 20-
19 40% ($p < 0.01$ to $p < 0.001$).

20

21 As shown in Figure 27, no significant differences were
22 recorded between passage 17 and 40 in terms of basal
23 insulin release and secretory responsiveness to glucose
24 and KCl. Thus all four human islet-derived cell lines
25 were able to maintain responsiveness ($p < 0.05$ to
26 $p < 0.001$) to glucose or KCl at passage 40. As shown
27 previously (Figure 9), cellular insulin content was
28 stable at increasing passage number.

29

30 Insulin-secretory characterisation studies have clearly
31 established that the four electrofusion-derived human
32 islet cell lines express many of the important

1 functional attributes of the parental B-cell. The
2 following studies provide a fundamental molecular
3 characterisation of the stimulus-secretion coupling
4 pathways in these new cell lines.

5
6 Glucose transporter activity is generally considered as
7 the first prerequisite for glucose-sensing in the
8 pancreatic B-cell (Tiedge et al., 1993). Presence of
9 glucose transporter (GLUT-1) was confirmed by Western
10 blot analysis. Membrane preparations of parental cells
11 (PANC-1 and Hup-T₃) and 1.1B4, 1.1E7, 1.4E7 and 1.2B4
12 cells were subjected to SDS-PAGE Western blotting
13 analysis using a sensitive antibody against the GLUT-1
14 protein. As shown in Figure 28, the antibody detected
15 a protein of approximately 48 kDa in each cell line.
16 However, whereas 1.1B4, 1.1E7, 1.4E7 and 1.2B4 cells
17 expressed significant levels of protein (particularly
18 1.1B4), the GLUT-1 transporter was not demonstrable in
19 parental PANC-1 nor Hup-T₃ cells.

20
21 After glucose transport, the next step in the B-cell
22 glucose-sensing mechanism involves the action of the
23 glucose phosphorylating enzyme, glucokinase (Lenzen and
24 Tiedge, 1994). Evaluation of glucokinase protein
25 expression in parental cells and hybrids cells using
26 Western blotting analysis is shown in Figure 29. By
27 using a specific antibody directed against the
28 glucokinase, a protein of 50 kDa was detected in each
29 cell line. No glucokinase expression was demonstrable
30 in parental PANC-1 and Hup-T₃ cells.

31

1 Further analysis of the glucose-sensing apparatus;
2 attempted to link molecular and functional aspects of
3 these novel insulin-secreting cell lines. The first
4 step involved the characterisation of the glucose
5 transport capacity of parental cells and each of the
6 cell lines.

7
8 Consistent with the presence of functional membrane
9 bound GLUT-1, 1.1B4, 1.1E7 and 1.4E7 cells exhibited
10 different 3-O-methyl-D-[1-³H]glucose (3-O-MG) transport
11 profiles at 1.1 and 16.7 mmol/l (Figure 30-32) compared
12 with parental cells (PANC-1). As shown in Figure 30-
13 32, the glucose uptake capacity was significantly
14 higher ($p < 0.05$ to $p < 0.001$) at 16.7 mmol/l glucose
15 compared with parental cells (PANC-1). When glucose
16 uptake was tested at 1.1 mmol/l glucose, only 1.1B4
17 cells recorded a significantly higher ($p < 0.05$) glucose
18 uptake capacity compared with PANC-1 cells (Figure 30).

19
20 A broadly similar pattern of glucose uptake profiles
21 was recorded in 1.2B4 cells (Figure 33). This cell
22 line exhibited different 3-O-methyl-D-[1-³H]glucose (3-
23 O-MG) transport profiles compared with parental cells
24 (Hup-T₃). At 16.7 mmol/l, glucose uptake capacity was
25 significantly higher in 1.2B4 cells compared with
26 parental cells (Hup-T₃). However, when the cells were
27 tested in 1.1 mmol/l 3-O-MG, no significant differences
28 were recorded.

29
30 Glucose transport properties of each novel human islet
31 cell line and parental cells were compared on the basis
32 of their initial velocity of 3-O-MG uptake. As shown in

1 Table 2, 1.2B4 cell line exhibited 6.5-fold increases
2 in the initial velocity at 16.7 mmol/l glucose compared
3 with 1.1 mmol/l glucose. Parental cells (Hup-T₃)
4 exhibited a 5.2-fold increase in velocity. In the
5 presence of 16.7 mmol/l glucose, 1.2B4 cells showed
6 significantly ($p < 0.05$) higher initial velocity uptake
7 compared with Hup-T₃ cell lines. However, when 3-O-MG
8 uptake was considered over 360 seconds, no significant
9 differences were recorded in the half-maximal
10 equilibration time either in 1.1 or 16.6 mmol/l 3-O-MG
11 (Table 2).

12
13 The initial velocity of 3-O-MG uptake by 1.1B4, 1.1E7
14 and 1.4E7 cells was 5.1, 6.1 and 5.4-fold higher at
15 16.7 mmol/l than 1.1 mmol/l, respectively (Table 2).
16 In comparison, PANC-1 cells exhibited 4.6-fold increase
17 in the initial velocity at 16.7 mmol/l glucose. At
18 both 1.1 mmol/l and 16.7 mmol/l, significant
19 differences in 3-O-MG uptake were recorded between
20 PANC-1 and 1.1B4 cells within the first 10 seconds. In
21 1.1E7 and 1.4E7 cells, significant differences ($p < 0.05$)
22 were only recorded at 16.7 mmol/l 3-O-MG. In terms of
23 half-maximal equilibration time within 360 seconds of
24 the measurements, only 1.1B4 showed a significant
25 difference ($p < 0.05$) (Table 3).

26
27 As shown in Figures 34 and 35, the parental immortal
28 partners and hybrid human islet cell lines showed
29 considerable differences in the relative contributions
30 of glukokinase and hexokinase to the total glucose
31 phosphorylating activity. Figure 34 shows the
32 percentage of hexokinase activity represented (mean \pm

1 sem, n=3) 98.5 ± 9.3 , 57.4 ± 4.6 , 57.4 ± 14.5 and 72.6
2 ± 5.3 % of the total activity obtained from PANC-1,
3 1.1B4, 1.1E7 and 1.4E7 cells, respectively. In Hup-T₃
4 and 1.2B4 cells, the percentage of hexokinase activity
5 represented (mean \pm sem, n=6) 45.2 ± 5.5 and 63.8 ± 9.9
6 %, respectively (Figure 35). The percentage glucose
7 phosphorylating activities of PANC-1, 1.1B4, 1.1E7 and
8 1.4E7 cells, attributed to glucokinase activity were
9 1.5 ± 0.8 , 42.6 ± 9.6 , 42.6 ± 11.3 and 27.4 ± 7.0 %,
10 respectively. In Hup-T₃ cells and derived clonal 1.2B4
11 cells, the percentage glucokinase activities were 54.7
12 ± 7.4 and 36.1 ± 6.4 %, respectively. With the
13 exception of 1.2B4 cells, all novel cell lines
14 exhibited significantly higher ($p < 0.01$ - 0.001)
15 glucokinase and lower ($p < 0.01$ to $p < 0.001$) hexokinase
16 (expressed as percentage) compared with PANC-1 cells.

17

18 Experiments conducted to assess the amount of
19 glucose being oxidised and utilised in the presence
20 of varying concentrations of glucose and 5-
21 thioglucose, showed interesting differences between
22 the parental cells and the derived human islet cell
23 lines.

24

25 Cell line 1.1B4 showed the highest levels of
26 oxidation of glucose peaking at 95.4 ± 4.5 pmol/1000
27 cells/h at 11.1mmol/l glucose (Figure 36). At
28 16.7mmol/l levels of glucose oxidation closely
29 resembled that at 5.6mmol/l. Inclusion of 5-
30 thioglucose was able to inhibit the oxidation of
31 glucose at both 1.1mmol/l and 16.7mmol/l glucose

1 (Figure 37). The utilisation of glucose by this
2 cell line followed a slightly different pattern with
3 the highest utilisation levels being recorded at
4 136.5 ± 1.7 pmol/1000 cells/h at 16.7mmol/l glucose
5 (Figure 38). 5-thiogluucose did not affect
6 utilisation at 1.1mmol/l glucose but was shown to
7 reduce utilisation at 16.7mmol/l glucose (Figure 38)

8
9 Oxidation of glucose by cell line 1.1E7 was maximal at
10 16.7mmol/l glucose giving a value of 60.4 ± 5.4
11 pmol/1000 cells/h (Figure 36). Unlike 1.1E7 cells, 5-
12 thiogluucose only marginally inhibited glucose oxidation
13 in 1.1B4 cells (Figure 37). However, the highest
14 levels of glucose utilisation were recorded in this
15 cell line, 181.9 ± 15.3 pmol/1000 cells/h at 16.7mmol/l
16 glucose and again 5-thiogluucose only affected
17 utilisation at 16.7mmol/l (Figure 38).

18
19 Cell line 1.4E7 resembled 1.1B4, showing highest
20 oxidation levels at 11.1mmol/l glucose with 5-
21 thiogluucose clearly inhibiting oxidation at both 1.1
22 and 16.7 mmol/l glucose (Figure 36 and 37). Utilisation
23 of glucose by this cell line peaked at 16.7mmol/l
24 glucose and was unaffected by inclusion of 5-
25 thiogluucose (Figure 38).

26
27 Cell line 1.2B4, cloned from parental Hup-T₃, showed
28 poor oxidation of glucose at all concentrations tested,
29 reaching only 7.0 ± 0.9 pmol/1000 cells/h at 16.7mmol/l
30 (Figure 37). 5-thiogluucose inhibited oxidation at
31 16.7mmol/l (Figure 40). Surprisingly, glucose
32 utilisation rates for 1.2B4 closely resembled those of

1 the other cell lines reaching a value of 127.5 ± 12.8
2 pmol/1000 cells/h at 16.7 mmol/l glucose (Figure 41).
3 5-thioglucoase had no affect on the utilisation of
4 glucose by this cell line.

5
6 Of the parental cell lines, glucose oxidation values
7 for PANC-1 were far higher than for Hup-T₃. PANC-1
8 cells showed highest oxidation levels at 11.1mmol/l
9 glucose with significant inhibition of oxidation at
10 both 1.1 and 16.7mmol/l. As with the clonal progeny
11 cell lines, PANC-1 glucose utilisation was highest at
12 16.7mmol/l - 163.7 ± 23.5 pmol/1000 cells/h. 5-thio-D-
13 glucose was also able to inhibit utilisation only at
14 this concentration. By contrast oxidation values for
15 Hup-T₃ closely resembled that for it's progeny cell
16 line 1.2B4, showing a maximum oxidation value of $12.6 \pm$
17 7.2 pmol/1000 cells/h at 11.1mmol/l glucose. In a
18 separate experiment 5-thioglucoase was able to inhibit
19 oxidation at both 1.1 and 16.7mmol/l glucose. Again
20 similar to 1.2B4, glucose utilisation values were
21 similar to those of the other cells lines reaching a
22 peak of 131.2 ± 13.2 pmol/1000 cells/h at 16.7mmol/l
23 glucose. 5-thioglucoase did not inhibit the utilisation
24 of glucose in Hup-T₃ cells.

25
26 Table 3 shows the relative metabolism flux through
27 oxidative glucose metabolism for the parental and each
28 of the derived human islet cell lines. The
29 significance of this index (ratio of glucose
30 oxidation:glucose utilisation) is that glucose
31 metabolism through oxidative pathway is most closely
32 linked to insulin secretion (Malaisse, 1992). Ratios

1 of all islet cell lines were significantly higher than
2 parental Hup-T₃ or PANC-1 cells (Table 4). 1.1B4,
3 1.1E7 and 1.4E7 cells showed a significant increase in
4 ratio on addition of 5-thioglucoase.

5
6 Glucokinase protein was detected in all 6 cell lines
7 studied and in contrast to insulin appeared as small
8 punctate spots of fluorescence scattered around the
9 cytoplasm (Figures 42). Antibody staining for this
10 enzyme did appear to differ in intensity the between
11 cell lines. Positive staining for insulin was detected
12 at high intensity levels in cell lines 1.1B4, 1.4E7
13 with lesser intensity in 1.1E7 and 1.2B4 (Figures 43).
14 From the images it can be seen that insulin protein is
15 located at various site throughout the cytoplasm,
16 appearing under fluorescence as diffuse bright patches.
17 In the parental cell lines (PANC-1 and Hup-T₃), no
18 cells out of the entire field showed any staining for
19 insulin. Similar to insulin staining, islet amyloid
20 polypeptide (IAPP) appeared as bright spots located to
21 the cytoplasm. Again all four cell lines showed a
22 positive signal for IAPP with higher levels of staining
23 in 1.1B4, 1.1E7 and 1.4E7 (Figures 44). No positive
24 staining was seen above background for either glucagon
25 or somatostatin in any of the cell lines tested
26 (Figures 45 and 46). Immunostaining characteristics of
27 all 6 cell lines is summarised in Table 5.

28

29 Each described embodiment describes the production and
30 characterization of hybrid human insulin-secreting cell
31 lines generated by the electrofusion technique.
32 Fusions were carried out using PANC-1 or Hup-T₃

1 immortal partner cells resulting in production of 38
2 hybrids (Figure 1). Of the 27 insulin-secreting clones
3 produced, the first four have been characterised
4 through a series of morphological and secretory
5 studies, analyzing the ability of each novel cell line
6 to respond to glucose and other regulators of β -cell
7 function. The four novel cell lines generated by
8 electrofusion, and characterised in this Chapter are
9 provisionally designated as 1.1B4, 1.1E7, 1.4E7 and
10 1.2B4 cells. Attempts to generate hybrids using TRM-1,
11 HAP-5 or B6 as immortal partner cells were thwarted by
12 failure of these cells to continue to proliferate in
13 tissue culture.

14

15 The three cell lines generated from PANC-1 (1.1B4,
16 1.1E7 and 1.4E7) and the cell line produced by
17 electrofusion with Hup-T₃ cells (1.2B4) all grew as
18 monolayers in tissue culture showing a pavemental
19 pattern when confluent, characteristic of epithelial
20 cells. The respective doubling times were
21 approximately 23 ± 1.7 hours for 1.1B4 cells, 23 ± 1.9
22 for 1.1E7 cells, 26 ± 1.5 hours for 1.4E7 cells and 36
23 ± 2.1 hours for 1.2B4 cells (n=4). These inherent
24 differences in the growth pattern support the view that
25 each represent a unique and novel clonal cell line.
26 The four cell lines were shown to express insulin,
27 glucokinase and IAPP by immunocytochemistry. No
28 visible staining was evident for glucagon or
29 somatostatin

30

31 Glucose is a principal regulator of insulin secretion
32 from the pancreatic B-cell (Meglasson and Matschinsky,

1 1986). Static incubation of the various novel human
2 islet cell lines over 60 min with a range of increasing
3 glucose concentrations revealed substantial differences
4 in insulin secretory responsiveness of each cell line.
5 1.1B4 cells showed a more appropriate stepwise pattern
6 of insulin output compared with other cell lines, with
7 a threshold at 5.6 mmol/l glucose. 1E7 and 1.4E7 cell
8 lines showed peak responses at 11.1 and 5.6 mmol/l
9 glucose, respectively. The falling off of insulin
10 output at higher glucose concentration possibly
11 reflects the dual action of glucose of increasing
12 intracellular Ca^{2+} sequestration and Ca^{2+} influx, as has
13 been implicated for the initial transient inhibitory
14 phase demonstrable in normal B-cells (Hellman *et al.*,
15 1992). This phenomenon is also observed in other
16 animal-derived insulin-secreting cell lines including
17 HIT-T15, BRIN-BG5 and BRIN-BG7 (Poitout *et al.*, 1996;
18 McClenaghan *et al.*, 1996b). In contrast, to the other
19 three electrofusion derived human islet cell lines, no
20 significant insulin secretory response to glucose was
21 observed in 1.2B4 cells under standard incubation
22 conditions.

23
24 The insulin output in response to glucose was
25 significantly enhanced in the presence of 200 $\mu\text{mol/l}$
26 IBMX in 1.1B4, 1.1E7 and 1.4E7 cells. The
27 phosphodiesterase inhibitor IBMX enhanced insulin
28 secretion by 20 to 60% and shifted the threshold to 5.6
29 mmol/l glucose in 1.1E7 cells. The action mediated by
30 IBMX indicates that the cells utilize IBMX to elevate
31 cAMP and potentiate Ca^{2+} -mediated insulin release as in
32 normal pancreatic B-cells (Hellman *et al.*, 1992). IBMX

1 also modestly enhanced insulin secretion from 1.1B4 and
2 1.4E7 cells in the absence of glucose. This
3 stimulatory effect most probably reflects the ability
4 of higher concentrations of cyclic AMP to stimulate
5 secretion through mobilization of intracellular Ca^{2+} and
6 promotion of Ca^{2+} influx, as also noted for the normal
7 pancreatic B-cell (Hellman, et al., 1992). Hup- T_3 -
8 derived 1.2B4 cells were insensitive to IBMX.

9
10 Inclusion of 2 mmol/l 5-thioglucoase, a glucose analog
11 that is a potent inhibitor of hexokinase, caused a
12 shift in glucose-insulin dose response for each cell
13 line. In 1.1B4, 1.1E7 and 1.2B4 cells, the effects were
14 significant ($p < 0.01$ to $p < 0.001$), consisting of a 20 to
15 50% reduction of insulin secretion at 0 mmol/l to 11.1
16 mmol/l glucose. In addition, inclusion of 5-
17 thioglucoase caused a shift in glucose responsiveness of
18 1.1E7 cells with a threshold concentration for
19 stimulation of insulin release at 5.6 mmol/l glucose.
20 In the presence of 5-thioglucoase, glucose-stimulated
21 insulin release also became apparent in Hup- T_3 -derived
22 1.2B4 cells. These observations suggest that the
23 beneficial effects of 5-thioglucoase on secretion are
24 due to inhibition of hexokinase with a greater
25 proportion of glucose flux catalyzed by glucokinase
26 such that the signaling potency of glucose metabolism
27 is increased (Hohmeier et al., 1997). Insulin output
28 and glucose responsiveness of 1.4E7 cells was not
29 affected by 5-thioglucoase.

30

31 Further characterisation of each cell line involved
32 evaluation of the insulintropic activity of a number

1 of amino acids including electrically neutral amino
2 acids (L-alanine, L-leucine) and cationic amino acids
3 (L-arginine). Although many studies have been
4 performed to elucidate the mechanism of amino acid-
5 induced insulin secretion (Henquin and Meissner, 1981;
6 Malaisse et al., 1991), the effects of many amino acids
7 including L-alanine and glycine are poorly understood.
8 When tested at a non-stimulatory (5.6 mmol/l) glucose
9 concentration, L-arginine significantly stimulated
10 insulin secretion in each of human islet-derived cell
11 lines. Significant effects of L-leucine and L-alanine
12 on insulin secretion at 5.6 mmol/l was observed only in
13 1.1B4 cells possibly reflecting that these amino
14 acids serve as potentiators rather than powerful
15 initiators of insulin release on human islet cells
16 (Yada, 1994).

17

18 When tested at 11.1 mmol/l glucose, each amino acid
19 tested, leucine, KIC, arginine and alanine caused
20 significant increases in insulin release. This
21 indicates that the novel cells possess each of the
22 various amino acid transport systems and related signal
23 recognition pathways described in normal pancreatic B-
24 cells (Hellman et al., 1971; Yada, 1994). Additionally
25 these findings demonstrate that the amino acids tested
26 can utilise stimulatory glucose for their
27 insulintropic actions on these novel human islet
28 cells. This confirms recent observations that amino
29 acids may act in a glucose-dependent manner, namely
30 that glucose acts as a fuel through its metabolism,
31 which may potentiate the uptake and utilization of
32 certain amino acids (McClenaghan and Flatt, 1999). It

1 is also interesting to note that glyceraldehyde, a
2 triose sugar, was able to significantly stimulate
3 insulin secretion from each of the novel cell lines.
4 Like glucose, glyceraldehyde stimulates insulin release
5 by its glycolytic metabolism and subsequent generation
6 of ATP (Halban and Wollheim, 1980). Depolarisation of
7 plasma membrane by a high concentration of KCl or
8 blockade of ATP-sensitive potassium (K^+ -ATP) channel
9 with glibenclamide or tolbutamide also provoked insulin
10 release from the novel cell lines as previously
11 established from normal B-cell (Kramer et al., 1996).
12

13 In order to study further the role of glycolysis and
14 K^+ -ATP channels in glucose-induced insulin secretion,
15 effects of diazoxide were evaluated. This agent is
16 known to open K^+ -ATP channels and thus acts to
17 repolarize the B-cell membrane and inhibit insulin
18 release (Henquin et al., 1992). As expected, this
19 agent significantly reduced insulin output of all four
20 cell lines at 16.7 mmol/l glucose, indicating the
21 importance of the K^+ -ATP channel in regulation of
22 insulin secretion from each of the clonal B-cell lines.
23

24 Ca^{2+} has been known for many years to play a important
25 role in nutrient-induced insulin secretion (Hellman,
26 1975). The importance of Ca^{2+} -influx in glucose-induced
27 insulin secretion was highlighted through incubations
28 performed in the absence of extracellular Ca^{2+} and with
29 the Ca^{2+} chelator EGTA. Depletion of Ca^{2+} significantly
30 inhibited insulin secretion at 16.7 mmol/l glucose from
31 each of the four cell lines. Blockage of voltage-
32 dependent Ca^{2+} channels (VDCC) using verapamil similarly

1 reduced glucose-induced insulin secretion. These
2 results clearly indicate the importance of Ca^{2+} and
3 voltage-dependent Ca^{2+} channels in the regulation of
4 insulin secretion from the novel hybrid cell lines. It
5 is therefore possible to infer from these functional
6 studies that the cells express K^{+} -ATP channels, SUR,
7 Kir and VDCC.

8
9 Equally importantly, insulin content and secretory
10 responses of all four human islet B-cell lines were
11 shown to remain stable at low and high passage number.
12 Thus cellular insulin content, basal insulin release
13 and secretory effects of glucose and KCl were similar
14 at passages 17 and 40. Such functional stability
15 appears to be a significant attribute of electrofusion-
16 derived compared with transfected pancreatic B-cell
17 lines.

18
19 Availability of molecular probes enabled Western
20 blotting analysis of all four novel human islet cell
21 lines and parental cell lines using a specific
22 antibodies directed against the GLUT-1 transporter
23 protein and against glucokinase. Proteins of
24 approximately 48 kDa and 50 kDa, respectively were
25 detected in all four cell lines but not in parental
26 PANC-1 nor Hup-T₃ cells. The GLUT-1 transporter is
27 known to play a important role in glucose uptake,
28 acting in conjunction with glucokinase to form the so
29 called B-cell glucose-sensing mechanism in pancreatic
30 B-cells (Shibasaki et al., 1990).

31

1 Measurements of GLUT-1 and glucokinase protein were
2 accompanied by functional measures of activity assessed
3 by evaluation of glucose transport and contribution of
4 glucokinase/hexokinase to the total glucose
5 phosphorylating activities of the various cell lines.
6 These studies revealed that the novel human islet
7 clones exhibited efficient glucose transport
8 characteristics which, unlike parental cells, were not
9 overwhelmed by increasing the extracellular glucose
10 concentration from 1.1 to 16.7 mmol/l. This
11 characteristic has previously been defined as a
12 distinguishing feature of the pancreatic B-cell
13 (Hellman et al., 1974). More recent molecular studies
14 consider glucose transport not to be rate limiting in
15 normal pancreatic B-cells except under most extreme
16 circumstances (Ohneda et al., 1994)).

17
18 Glucokinase is generally believed to be one of the most
19 important determinants of glucose sensitivity in the
20 pancreatic B-cell (Lenzen and Tiedge, 1994). In
21 particular, a high contribution of glucokinase to total
22 phosphorylating activity (as apposed to hexokinase) is
23 a landmark feature of normally functioning insulin-
24 secreting cells. In the present study, glucokinase
25 contributed < 2% to the glucose phosphorylating
26 activity of parental PANC-1 cells. Consistent with
27 Western blotting data, the glucokinase contribution of
28 derived 1.1B4, 1.1E7, 1.4E7 ranged between 27-43%. The
29 other cell line, 1.2B4 was characterised by 36%
30 contribution of glucokinase to total phosphorylating
31 activity. These measures compare quite favorably to
32 the value of 50% reported to the normal B-cells

1 (Lenzen, 1990), and clearly indicate efficient
2 transport and metabolism of glucose in the human
3 partner B-cells.

4
5 Metabolism of glucose by the B-cell represents a
6 culmination of glucose transport and phosphorylation
7 activities. Evaluation of the metabolic response in
8 parental and derived cell lines indicated a stepwise
9 increase in glucose oxidation/utilisation with
10 increasing glucose concentration. Inhibition of
11 hexokinase activity using 5-thioglucoſe also showed
12 inhibitory effects as reported elsewhere (Hohmeier et
13 al., 1997). Since glucose stimulation of insulin
14 secretion is tightly linked to ATP generation, relative
15 flux of glucose metabolism through oxidative pathways
16 has been considered as a particularly noteworthy
17 indicator of the functional integrity of the pancreatic
18 B-cell (Malaisse, 1992). Calculation of the ratios
19 glucose oxidation:glucose utilisation revealed higher
20 values in each of the hybrid cell compared with
21 parental PANC-1 or Hup-T₃ cells. Such effects were
22 broadly linked to relative glucokinase activities of
23 the various cell lines.

24
25 Although the immunohistochemistry results were only
26 part of early studies on the expression of B-cell
27 markers, it is reassuring that positive staining for
28 insulin was detected by the insulin antiserum in all of
29 the clonal hybrid cell lines. As seen from the FITC
30 images cells do not appear to have uniform insulin
31 staining. This probably reflects the inability of this
32 particular approach to detect low levels of insulin

1 present in some of the cells. Staining for insulin
2 using the same antibody in rat pancreas showed strong
3 fluorescent signal (results not shown) but monolayers
4 of B-cells contained far lower levels of insulin,
5 estimated less than 5% of normal pancreatic islet
6 cells.

7
8 Glucokinase protein was detected by immunocytochemistry
9 in all cell lines as small, granular spots of
10 fluorescence scattered around the cytoplasm. As with
11 insulin it was difficult to visually quantify the
12 levels of glucokinase from these experiments, however a
13 careful examination of each cell line indicated that
14 levels of positive staining were again augmented in
15 hybrid islet cells compared with the parental cell
16 lines. IAPP was found in all cell lines and also
17 appeared as small spots of positive staining around the
18 cytoplasm and was less intense than the staining seen
19 for glucokinase. No positive staining for either
20 glucagon or somatostatin was seen in any of the cell
21 lines tested supporting the view that the hybrids
22 represent a pure population of clonal pancreatic B-
23 cells.

24
25 The present invention reports the generation and
26 characterisation of four novel electrofusion-derived
27 human insulin-secreting pancreatic B-cell lines.
28 Functional assessment of these unique cells
29 indicates that many of the features of the normal
30 human pancreatic B-cell have been inherited by the
31 hybrid cells from the human donors (Table 6).

32

1 DNA identity profiling of the four electrofusion
2 derived human insulin-secreting pancreatic B cell
3 lines indicates that cell lines 1.1E7, 1.4E7 and
4 1.1B4 have similar DNA profiles which 1.2B4 has a
5 different profile.

6

7 Tables 7,8,9 and 10 show the identity profile
8 results of 1.1E7, 1.4E7, 1.1B4 and 1.2B4
9 respectively.

10

11 These profiles indicate that cell lines produced by
12 the electrofusion process which show features of
13 normal pancreatic B cells do not require to share
14 identical genetic identity.

15

16 The cell lines produced appear to have superior
17 functional qualities to many existing animal cell
18 lines which can be attributed to presence of key
19 components of the normal pancreatic B-cell stimulus-
20 secretion coupling pathway. These various
21 observations, combined with functional stability,
22 indicate that electrofusion-derived human pancreatic
23 B-cell clones will be of substantial benefit for
24 diabetes research. In addition, these cell lines
25 will enable the use of cellular engineering using
26 electrofusion-derived pancreatic B-cells to provide
27 therapy for type 1 diabetes.

1 **CLAIMS**

2

3 1. A human pancreatic cell line produced by
4 electrofusion of normal human islet cells with cells
5 from at least one immortal human cell line wherein
6 the human pancreatic cell-line is capable of
7 secreting insulin.

8

9 2. A human pancreatic cell line capable of
10 secreting insulin chosen from the group of cell
11 lines consisting of the cell-line deposited under
12 Accession No 00112811 at the European Collection of
13 Cell Cultures (ECACC), CAMR, Salisbury, Wiltshire on
14 28 November 2000 and the cell-lines deposited under
15 Accession Nos PTA 3523, PTA 3524 and PTA 3525 at the
16 American Type Culture Collection, 10801 University
17 Boulevard, Manassas, Virginia 20100-2209, USA on 17
18 July 2001.

19

20 3. A process for the production of human
21 pancreatic cell lines capable of secreting insulin,
22 the process including the steps of electrofusing a
23 mixture of normal human islet cells with cells from
24 at least one immortal human cell line and incubating
25 the mixture to generate hybrid cells.

26

27 4. The process as claimed in claim 3 wherein the
28 human islet cells and immortal human cells are mixed
29 in a 1:1 ratio.

30

31 5. The process as claimed in claims 3 or 4 wherein
32 electrofusion occurs in a helical chamber.

1 6. The process as claimed in any of claims 3 to 5
2 wherein the electrofusion step includes exposing the
3 cells to a first pulse phase of AC field, a second
4 pulse phase of DC field and a third pulse phase of
5 AC field.

6

7 7. The process as claimed in claim 6 wherein the
8 first pulse phase is comprised of 7V, 2MHZ of AC
9 field for 30 seconds.

10

11 8. The process as claimed in claims 6 or 7 wherein
12 the second pulse phase is comprised of 60V, triple
13 pulses of DC field with each of the triple pulses
14 being 15 seconds in duration.

15

16 9. The process as claimed in claim 6, 7 or 8
17 wherein the third pulse phase is comprised of 7V,
18 2MHZ of AC field for 30 seconds.

19

20 10. The process as claimed in any of claims 3 to 9
21 wherein the media in which the cells are incubated
22 comprises hypoxanthine, aminopterin and thymidine.

23

24 11. The process as claimed in claim 10 wherein
25 hypoxanthine is present in the incubating media at a
26 concentration of between 0.05 μ mol/l to 0.5 μ mol/l.

27

28 12. The process as claimed in claim 10 or 11
29 wherein aminopterin is present in the incubating
30 media at a concentration of between 0.2-0.6 μ mol/l.

31

1 13. The process as claimed in claims 10, 11 or 12
2 wherein thymidine is present in the incubating media
3 at a concentration of between 10-20 $\mu\text{mol/l}$.
4

5 14. The process as claimed in any of claims 3 to 13
6 wherein incubation is carried out in the presence of
7 at least one secretagogue chosen from the group
8 comprising glucose, glyceraldehyde, arginine,
9 leucine and alanine.
10

11 15. The process as claimed in any of claims 3 to 14
12 wherein incubation is carried out in the presence of
13 at least one substance chosen from the group
14 comprising KCl, IBMX, thioglucose, tolbutamide,
15 diazoxide and verapamil.
16

17 16. A cell line produced by a process as claimed in
18 any of claims 3 to 15 which exhibits glucose
19 transport characteristics as efficient as normal
20 pancreatic B cells.
21

22 17. A cell line produced by a process claimed in
23 any of claims 3 to 16 which exhibits glucose
24 phosphorylating activity consistent with normal
25 pancreatic B cells.
26

27 18. The use of insulin producing cells produced by
28 the process as claimed in any of claims 3 to 15 to
29 provide gene therapy for type 1 diabetes.
30

31 19. The use of insulin producing cells produced by
32 the process as claimed in any of claims 3 to 15 in

1 the preparation of a medicament for the treatment of
2 diabetes.

3

4 20. Use of a cell-line as claimed in any of claims
5 1, 2, 16 or 17 in the preparation of a medicament
6 for the treatment of diabetes.

7

8 21. Use of a cell-line as claimed in any of claims
9 1, 2, 16 or 17 for the production of insulin.

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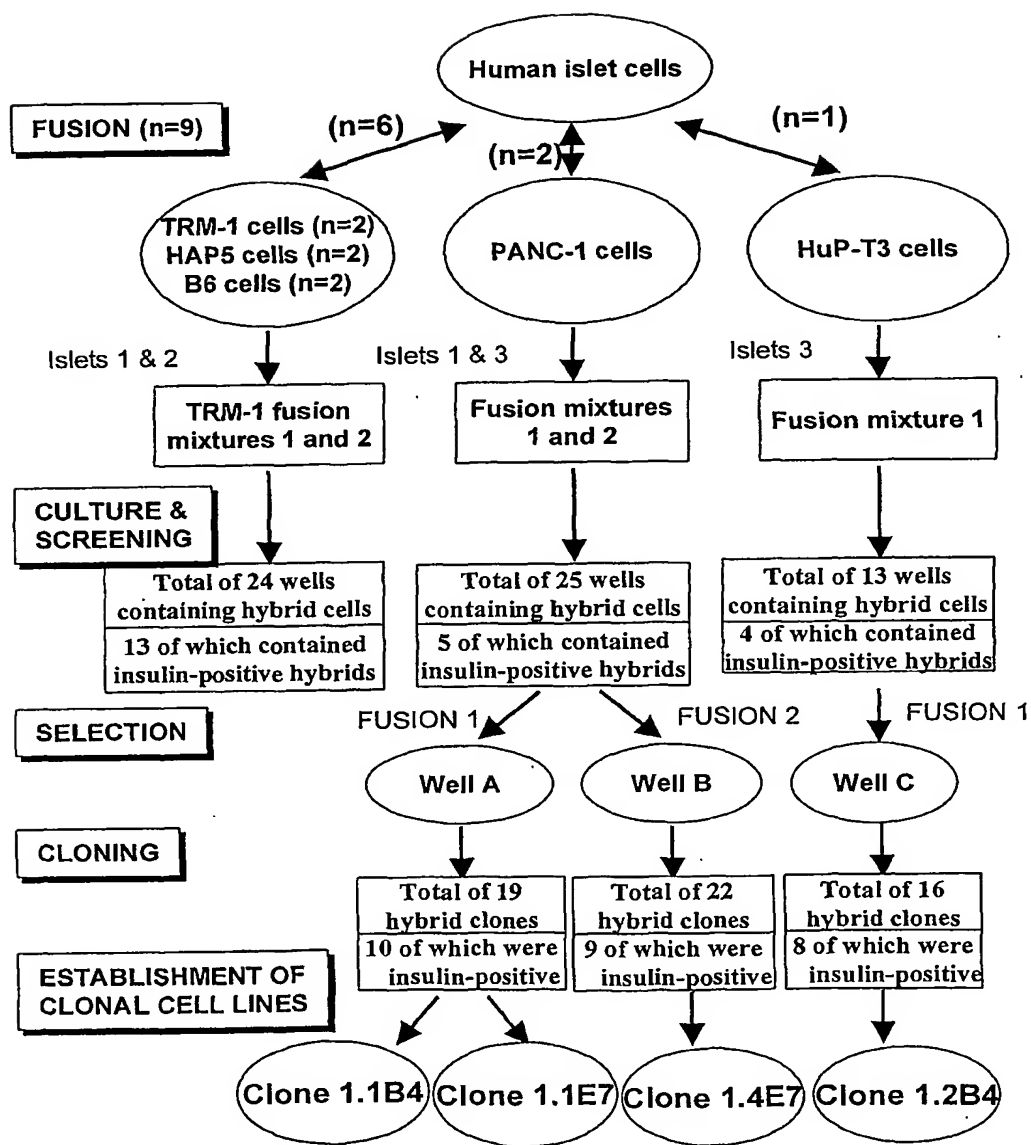


Figure 1

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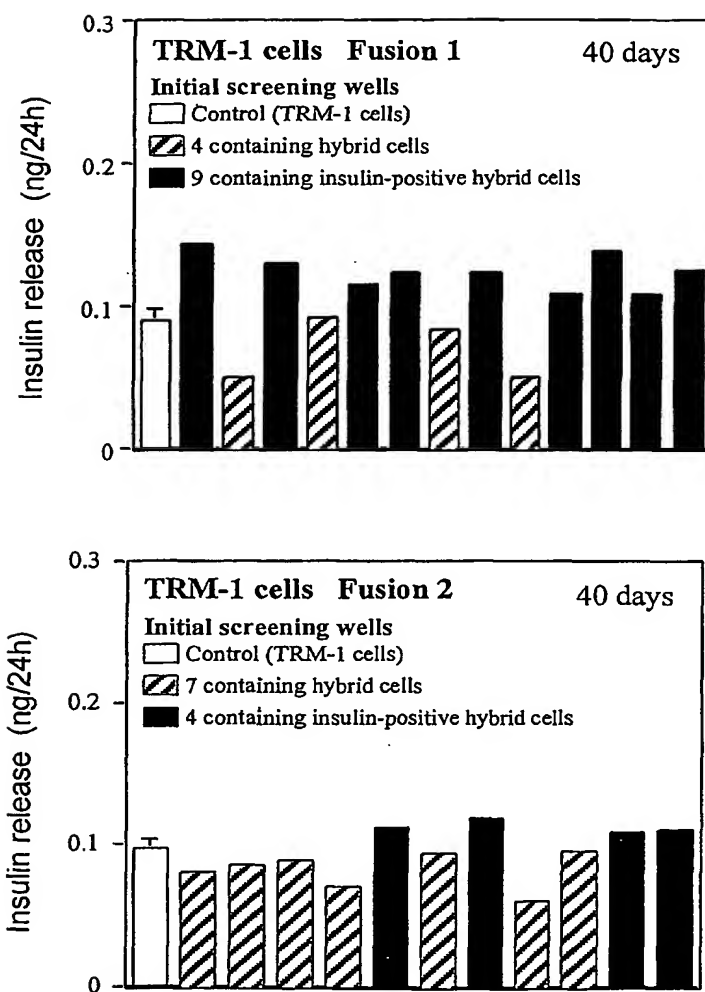


Figure 2

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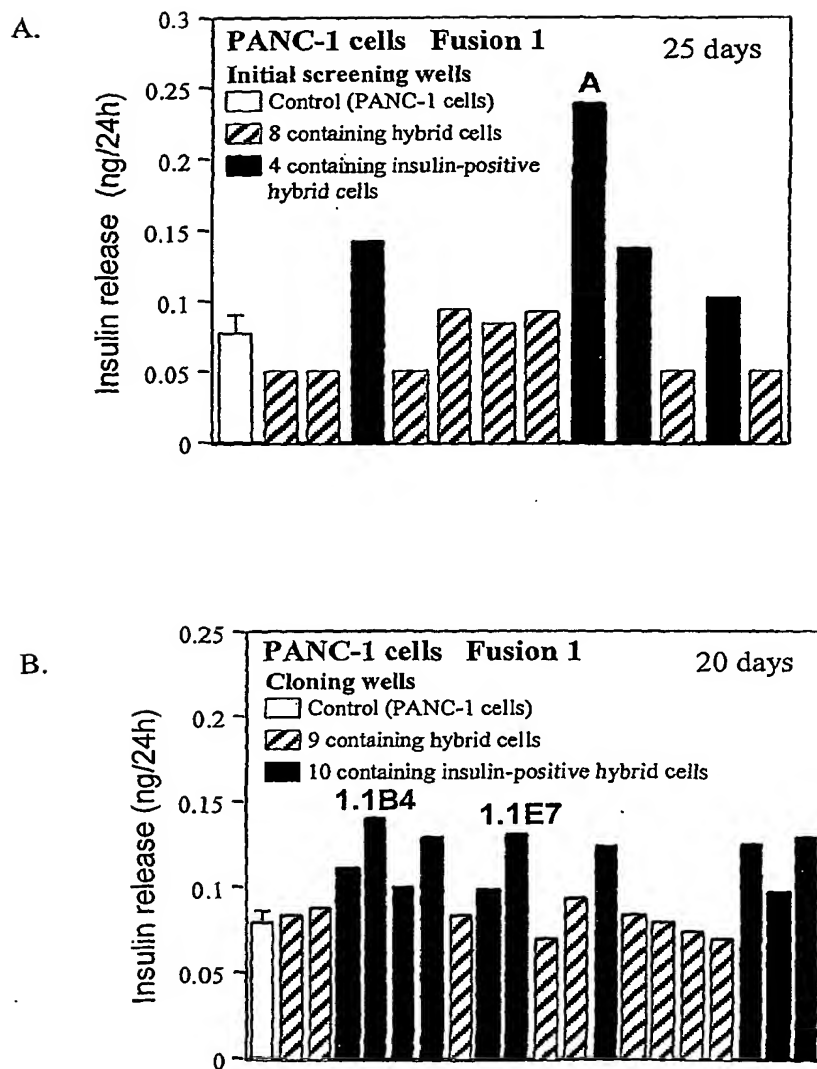


Figure 3

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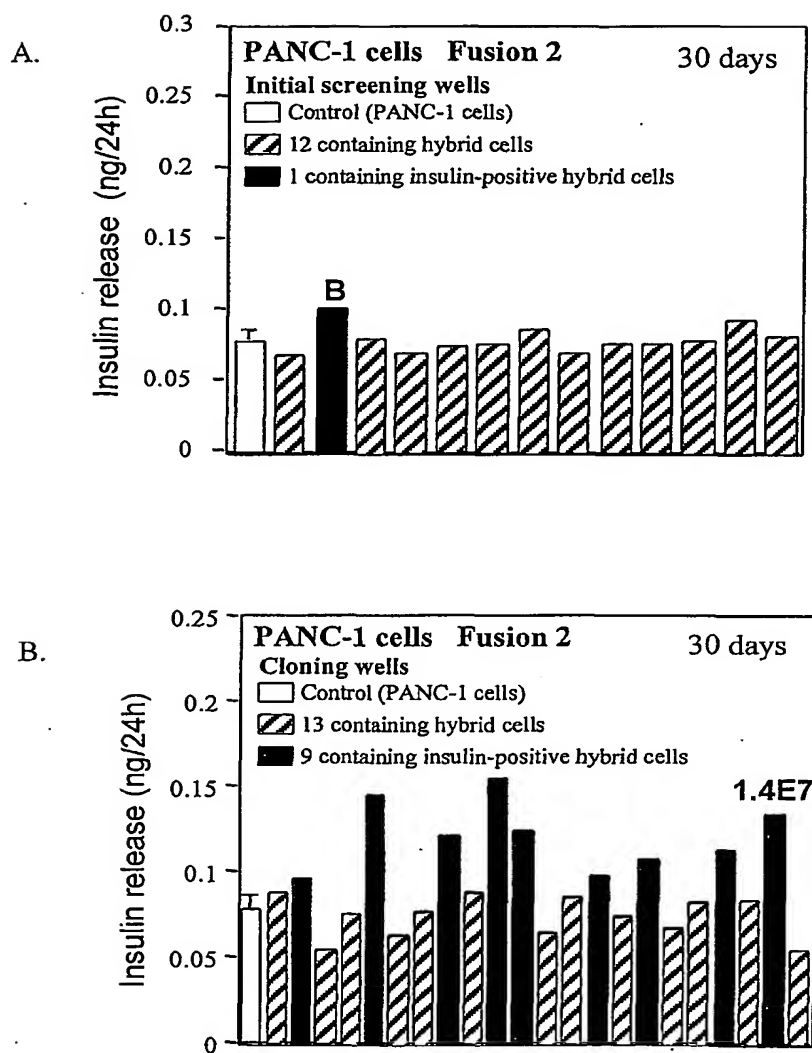


Figure 4

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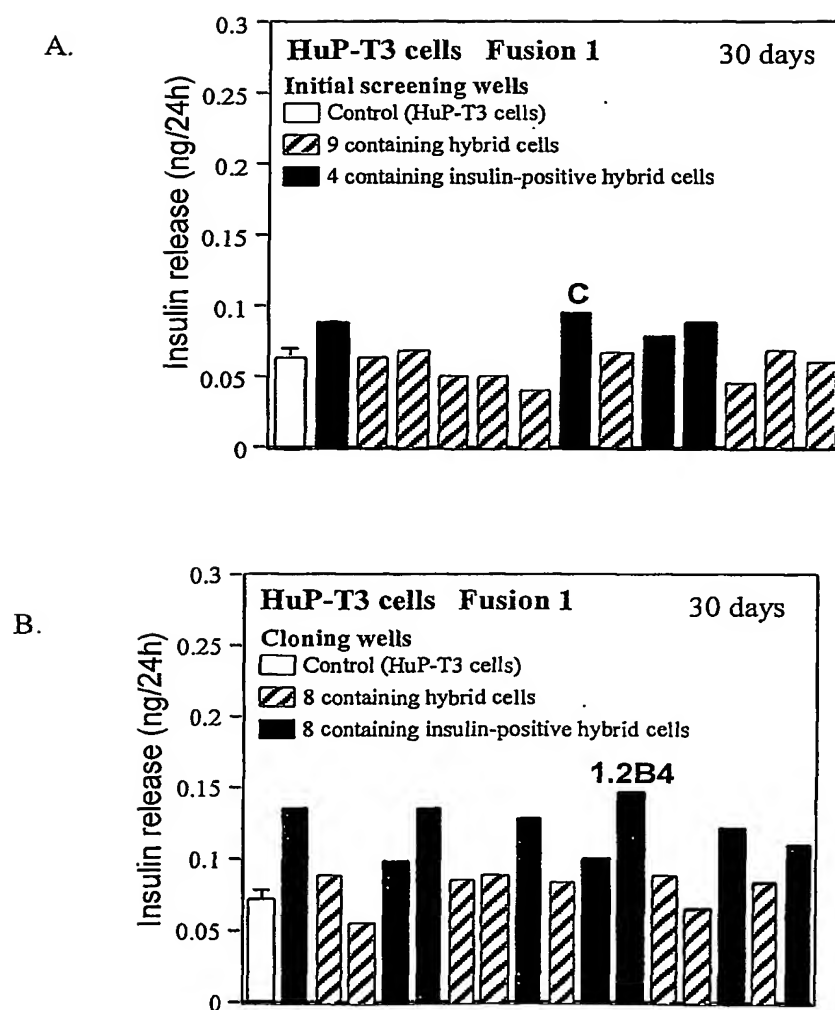
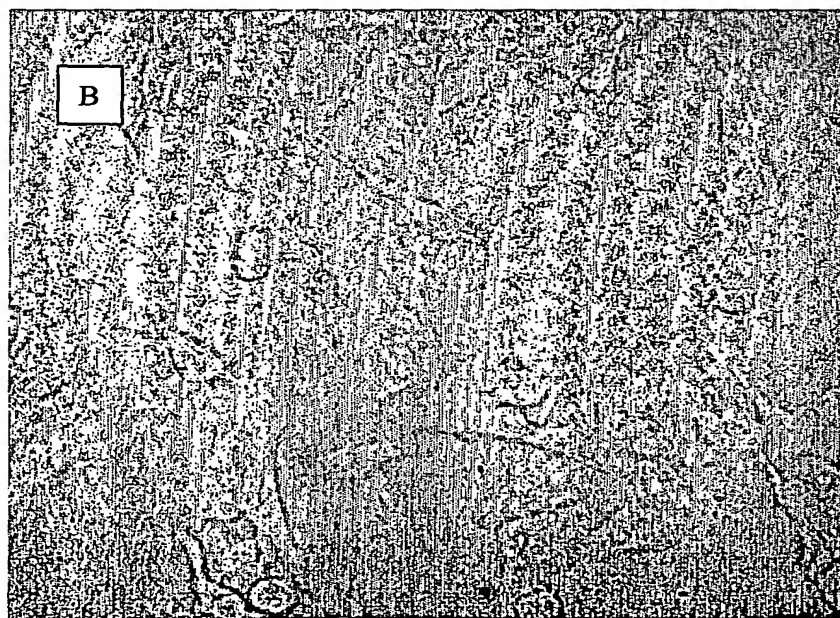
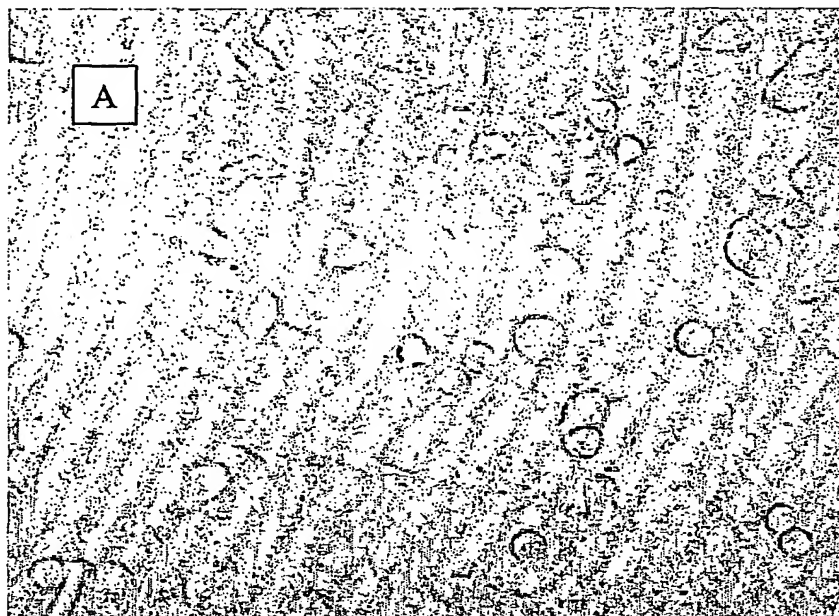


Figure 5

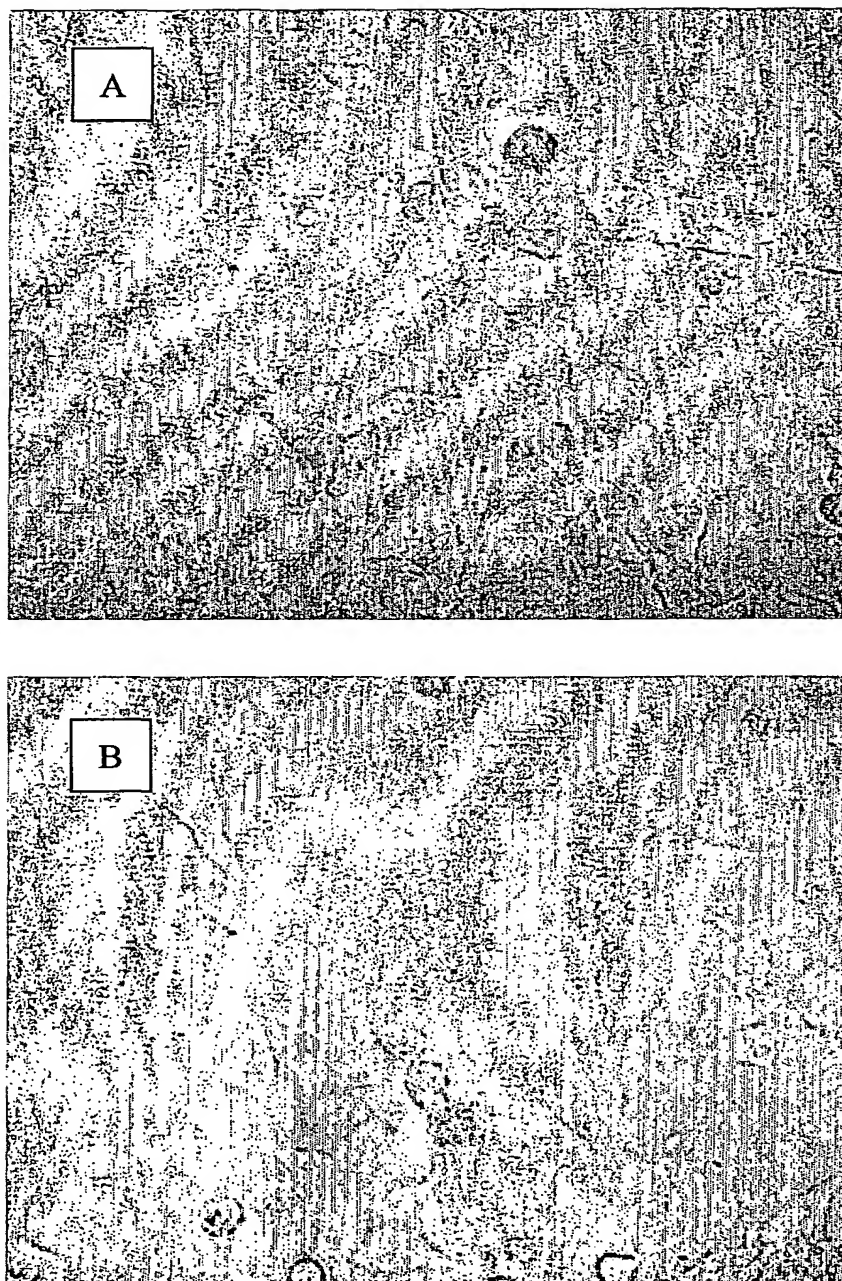
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Figure 6



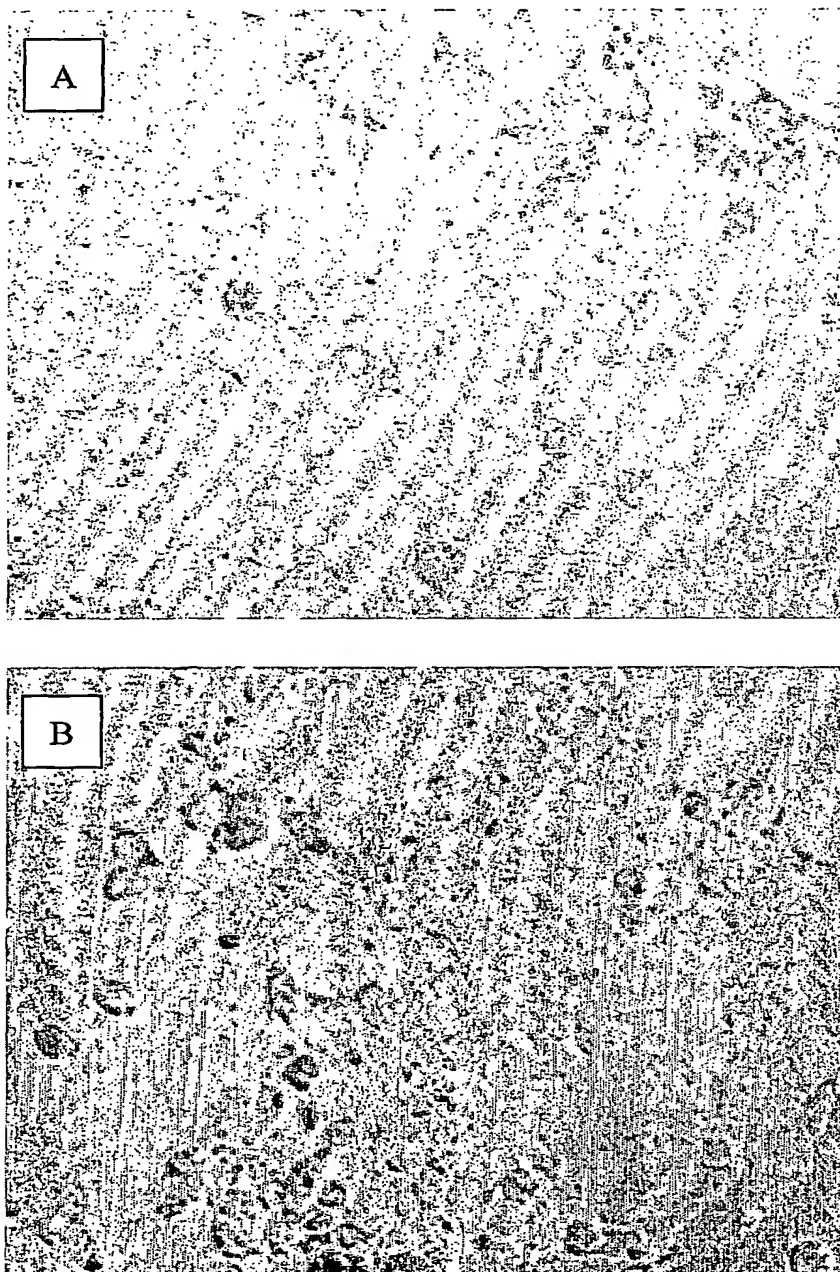
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Figure 7



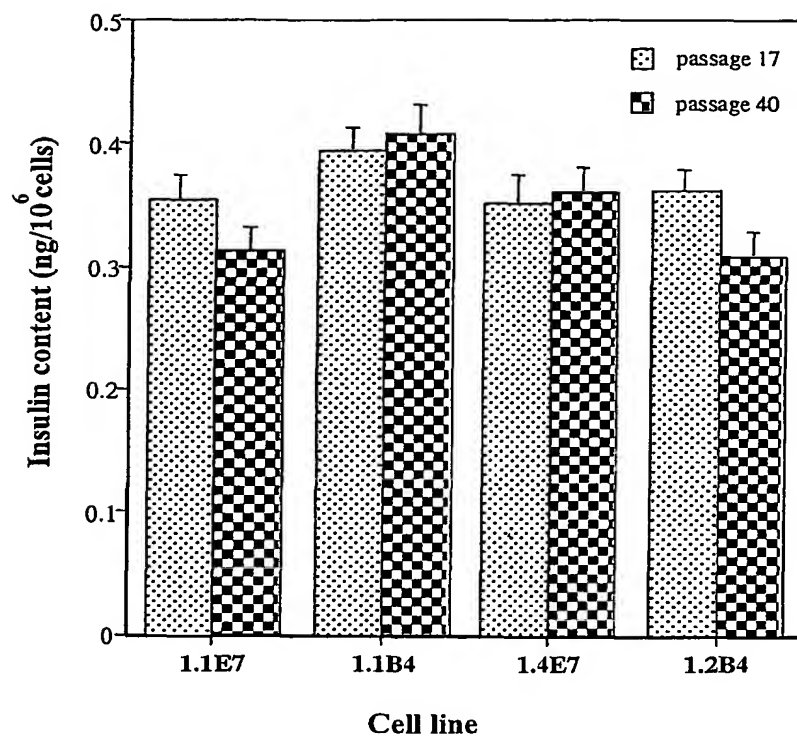
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Figure 8



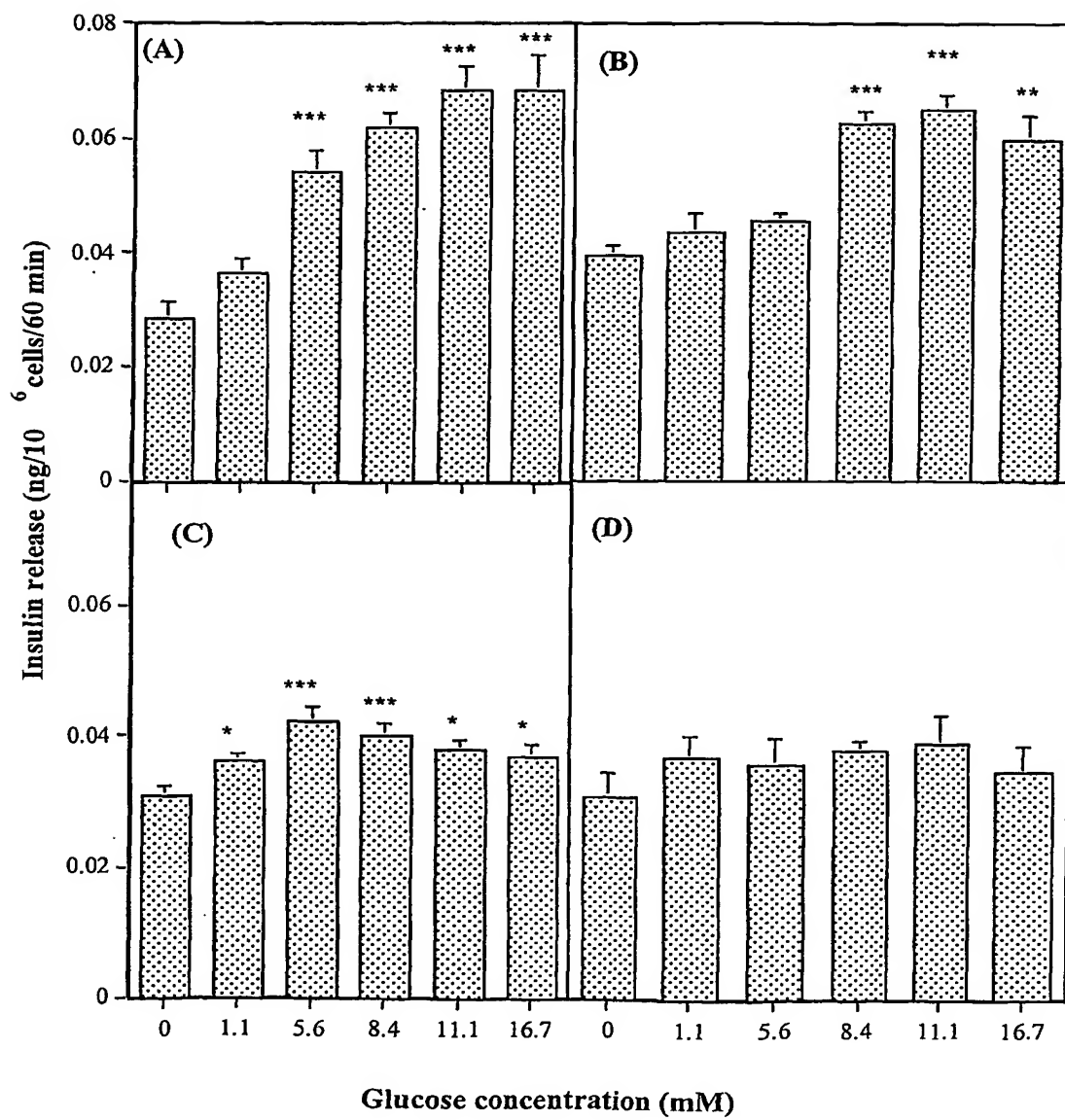
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Figure 9



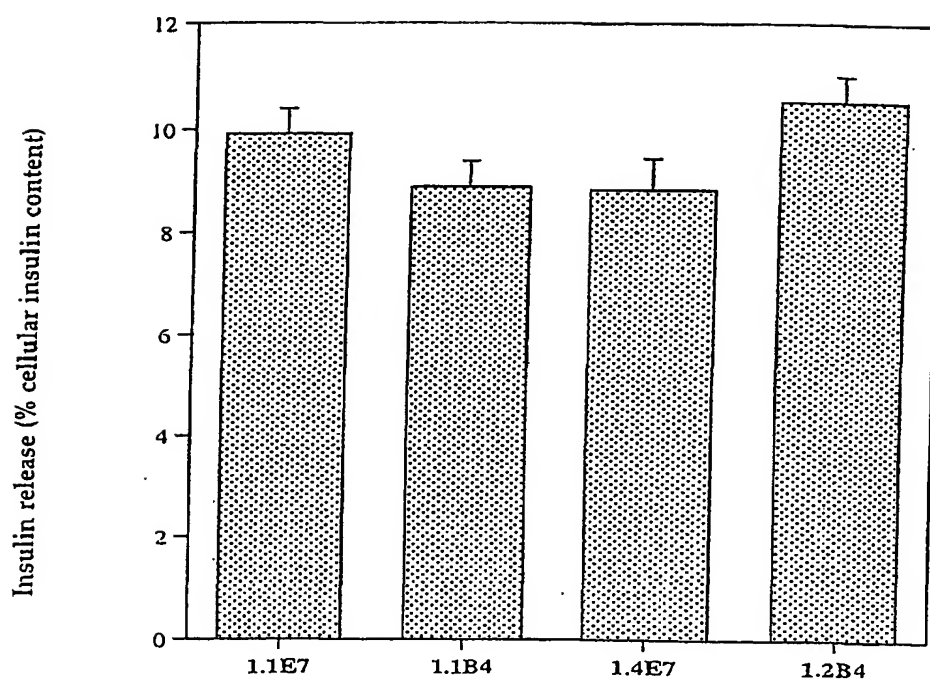
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Figure 10



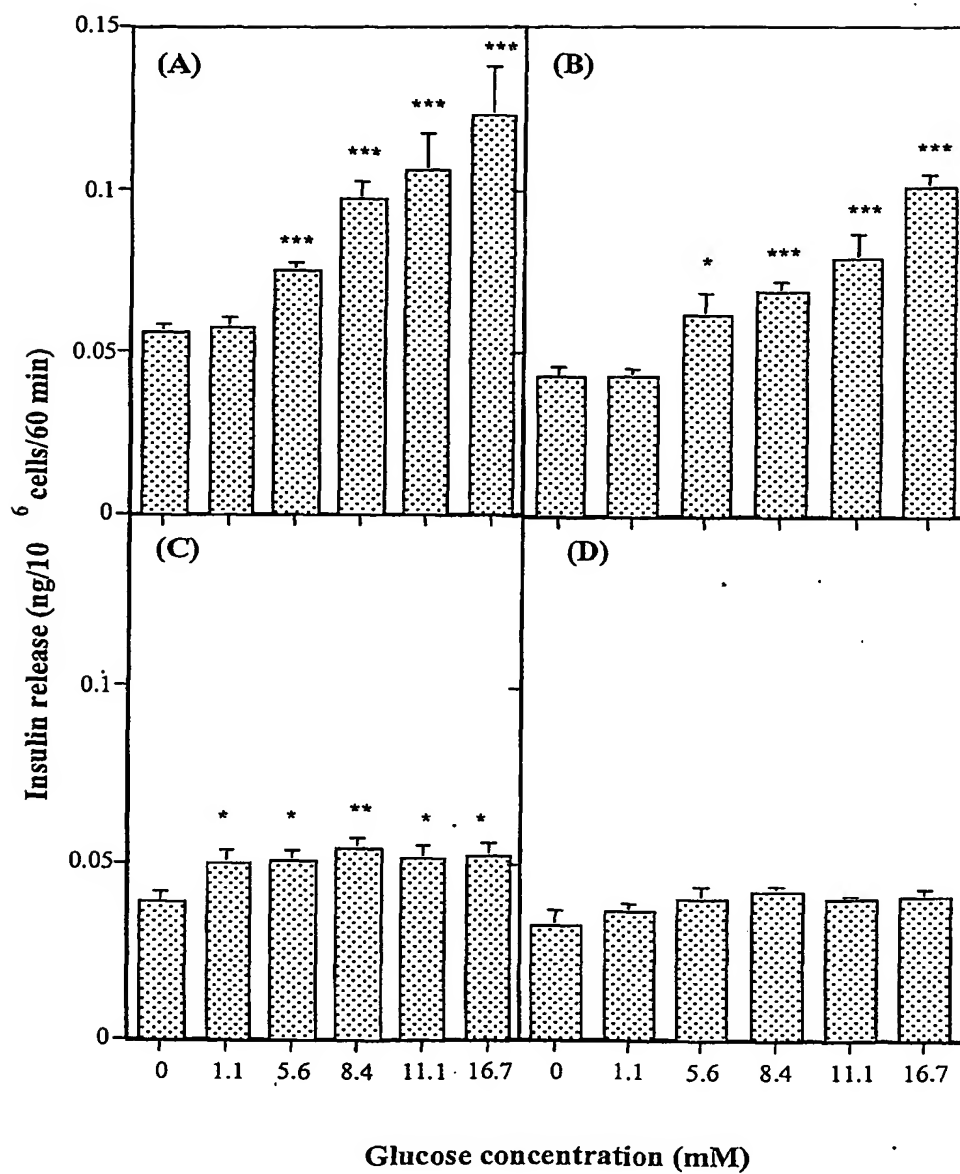
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Figure 11



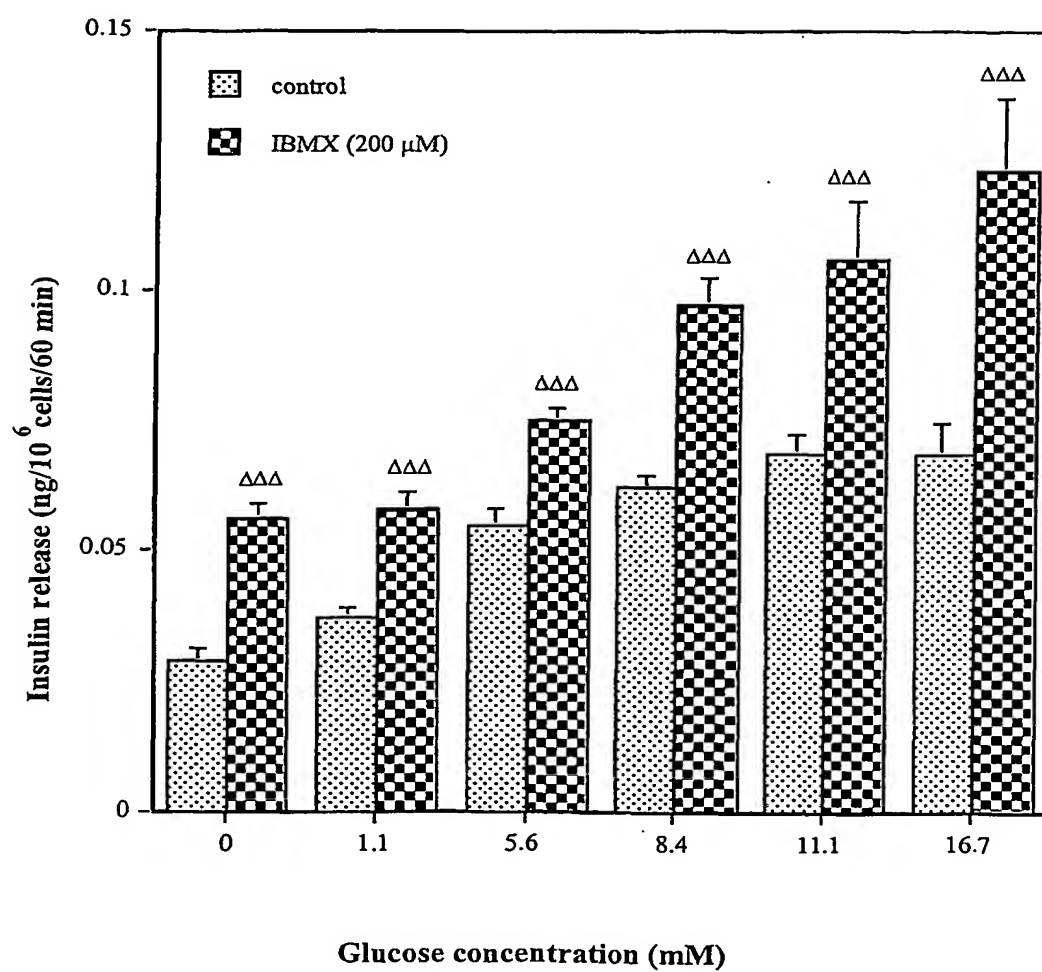
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Figure 12



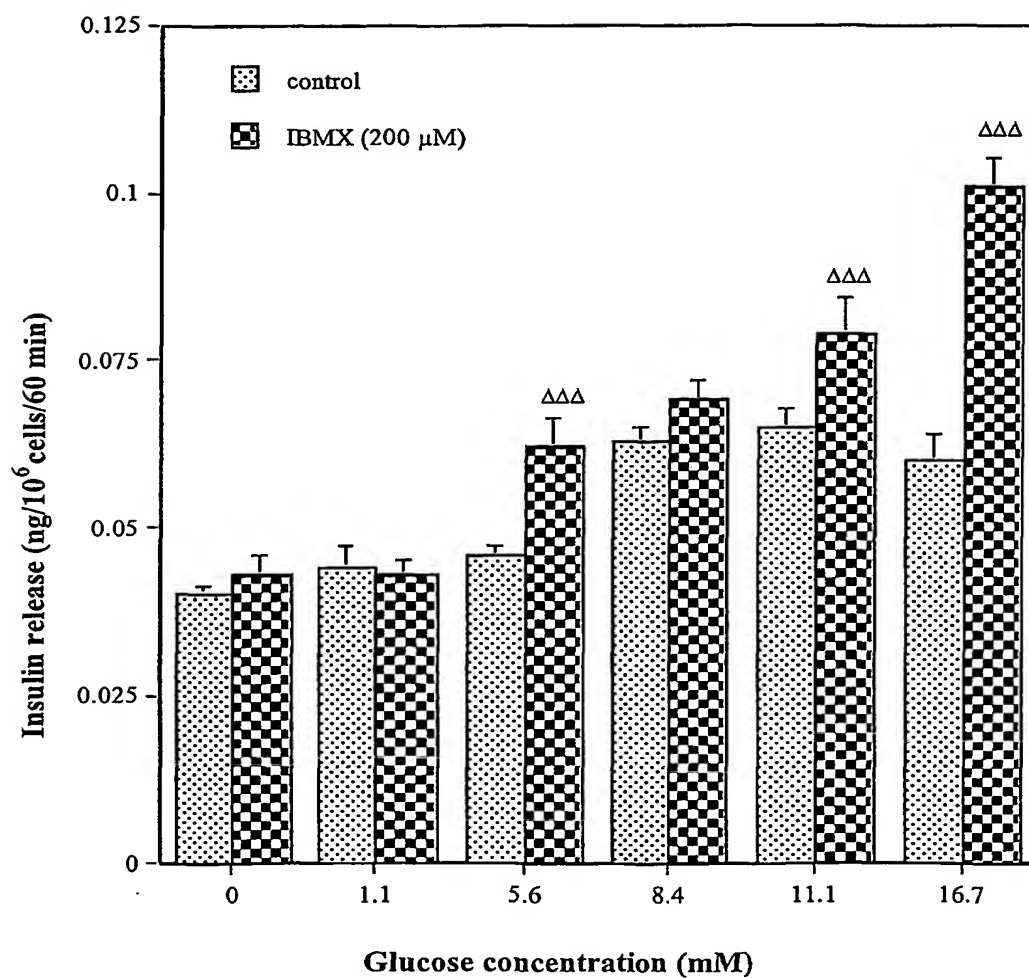
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Figure 13



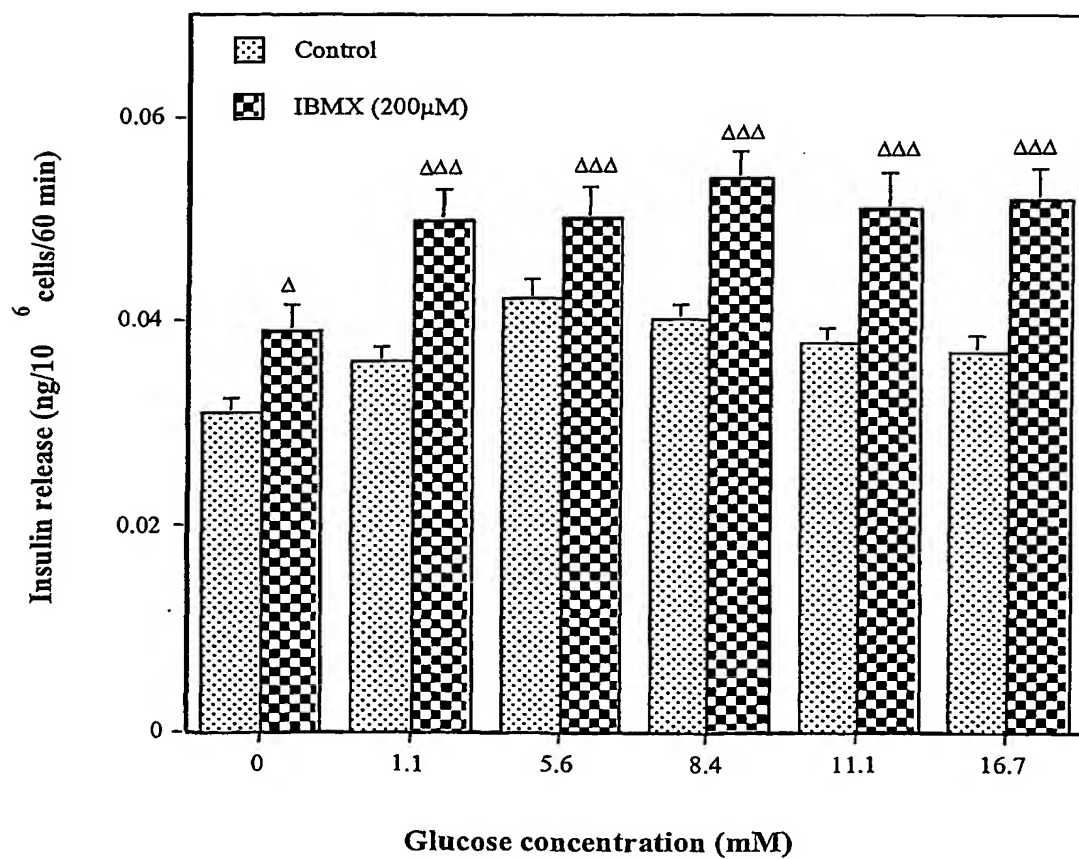
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Figure 14



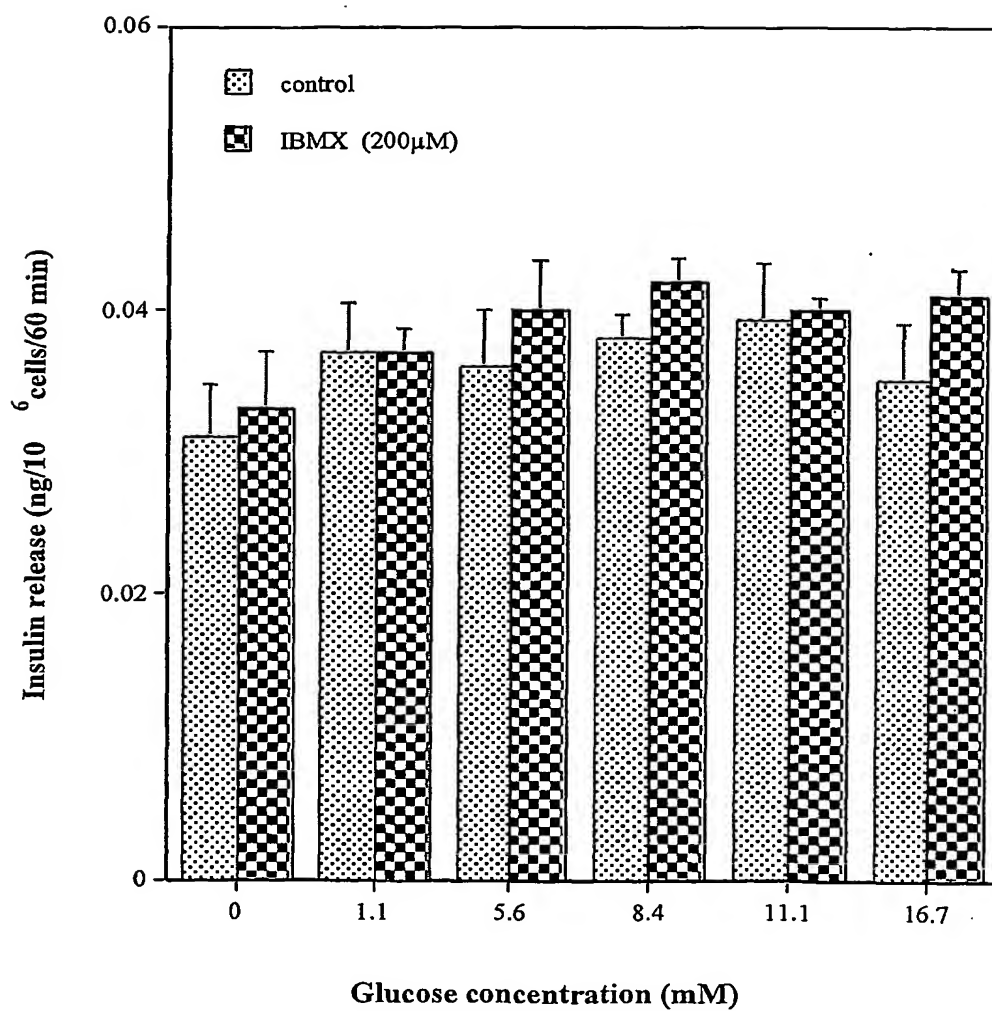
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Figure 15



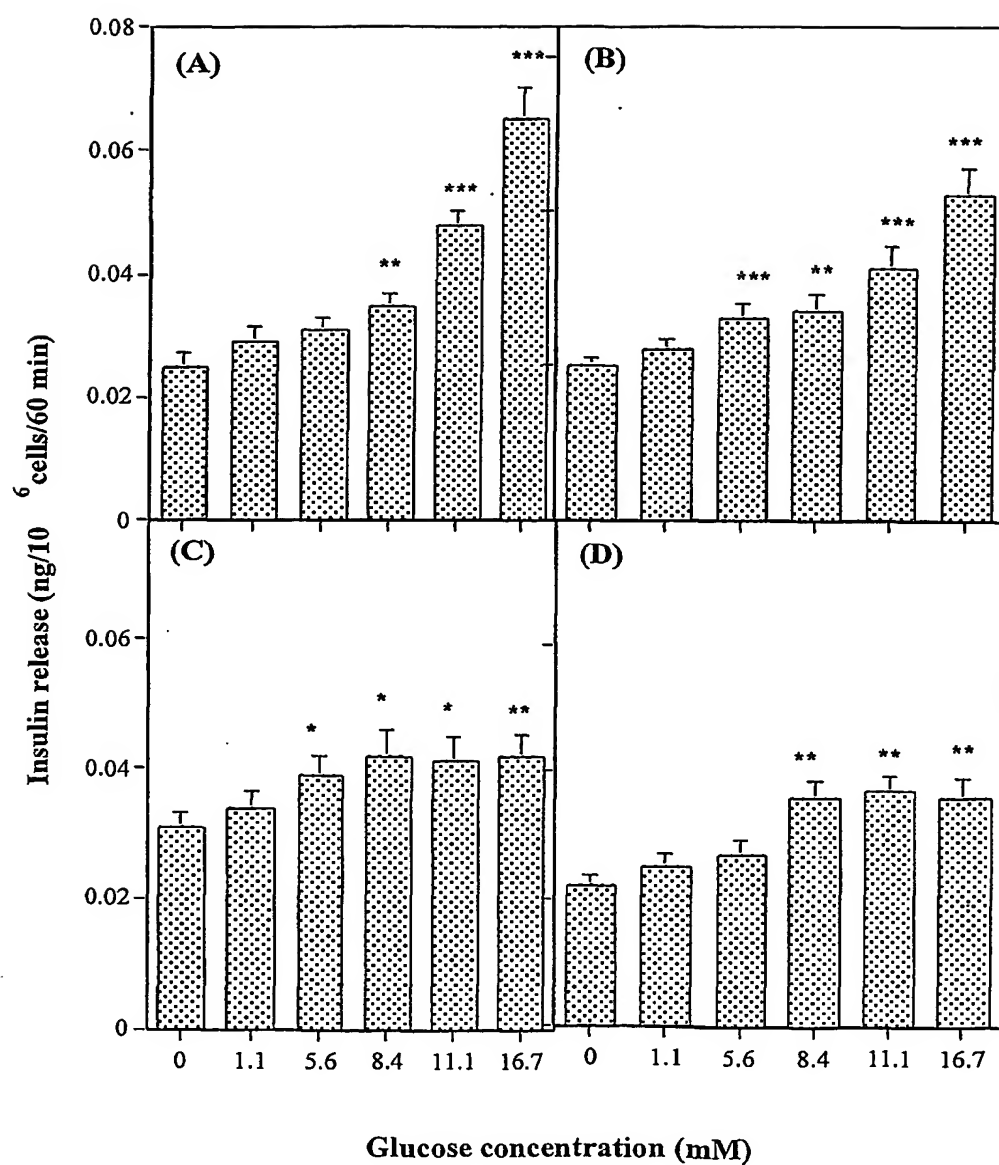
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Figure 16



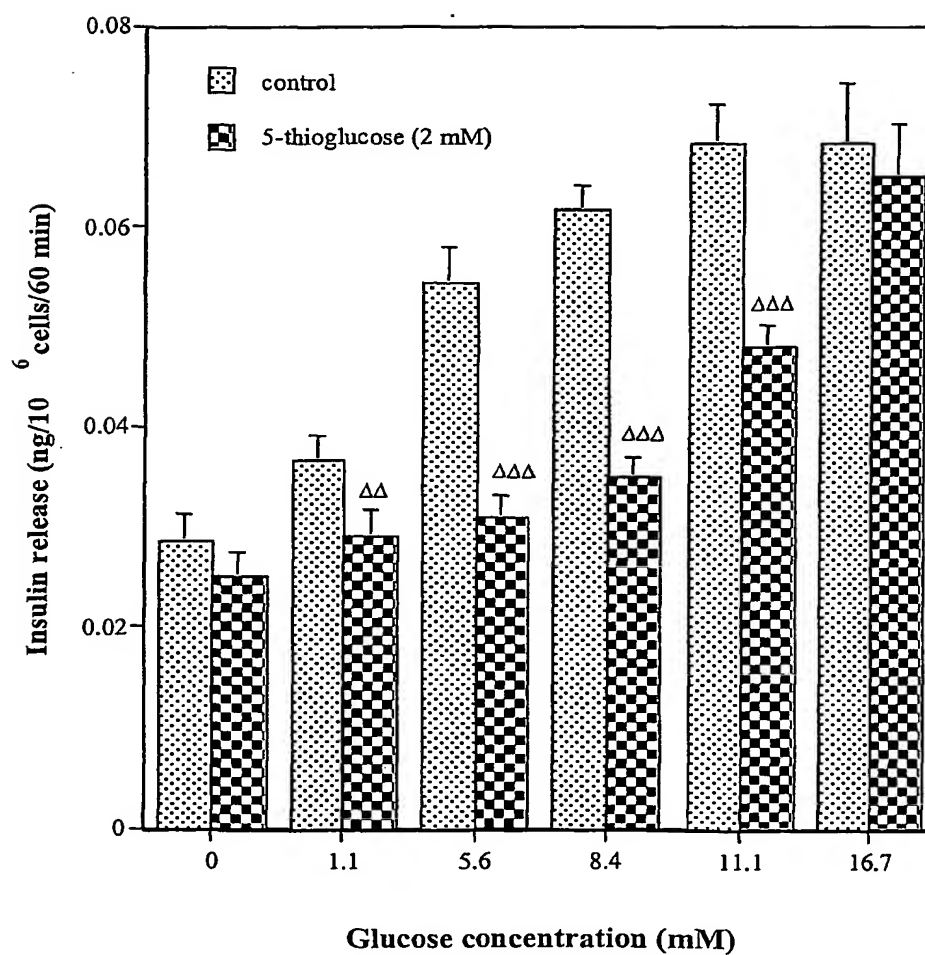
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Figure 17



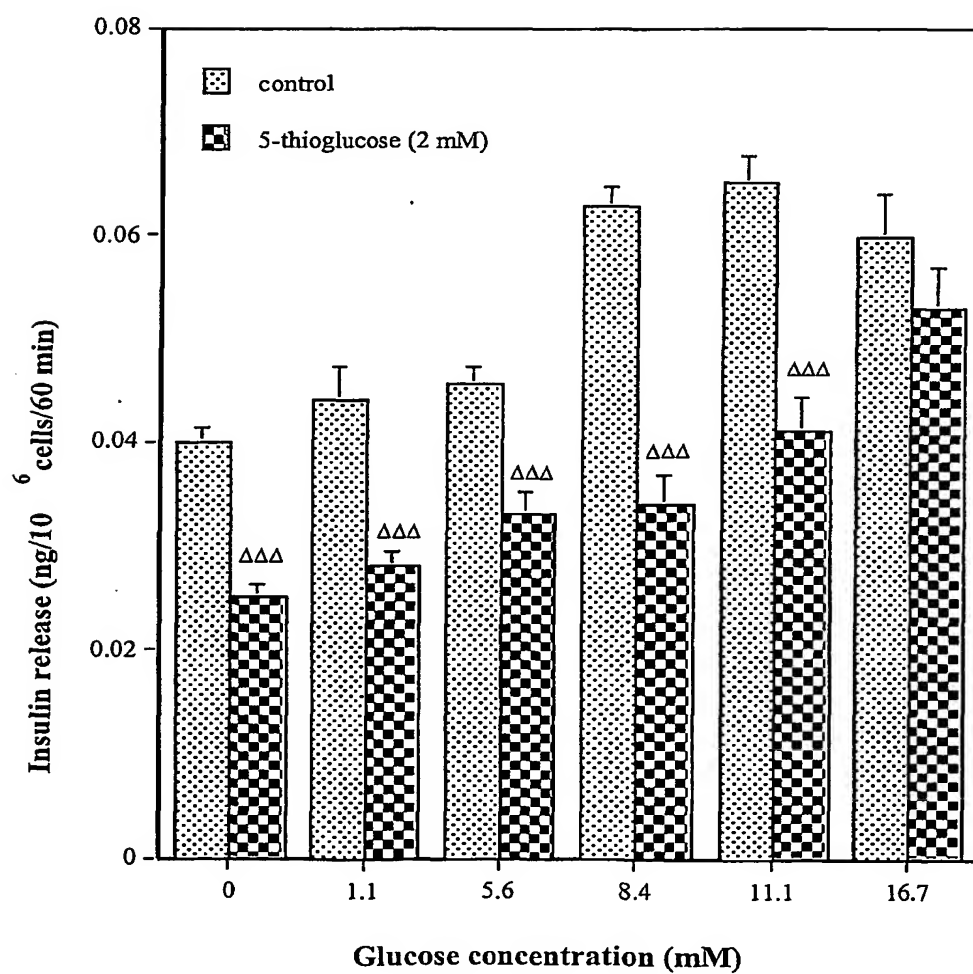
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Figure 18



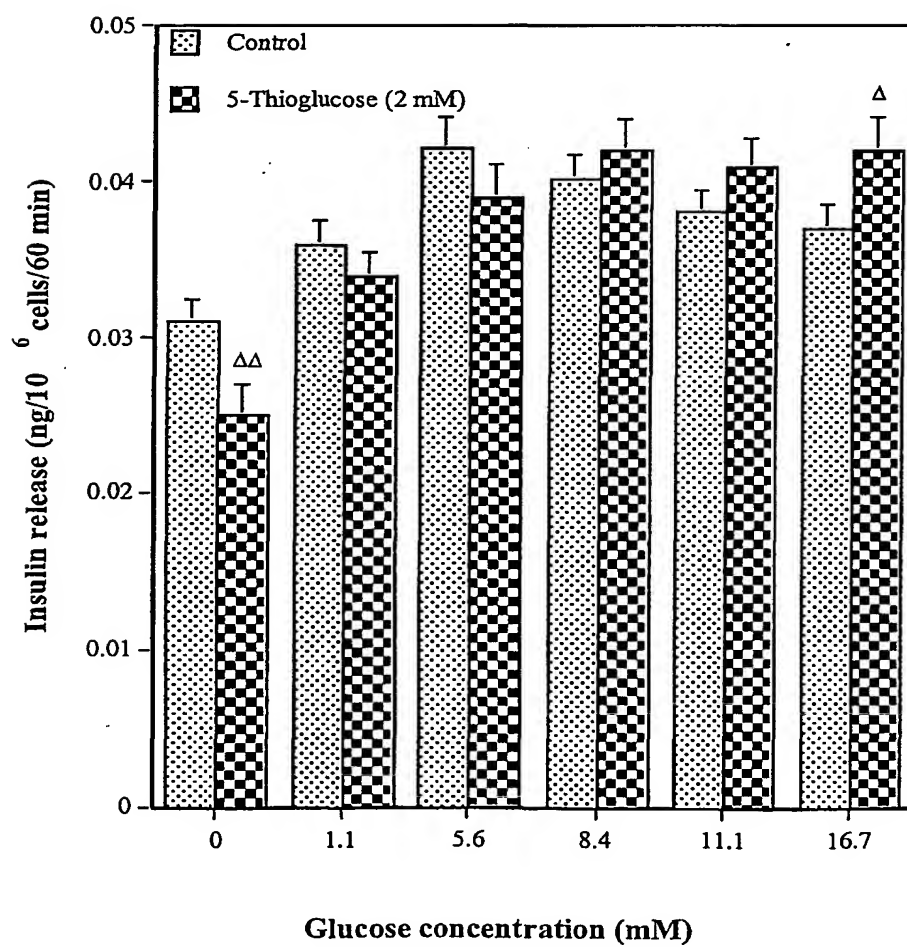
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Figure 19



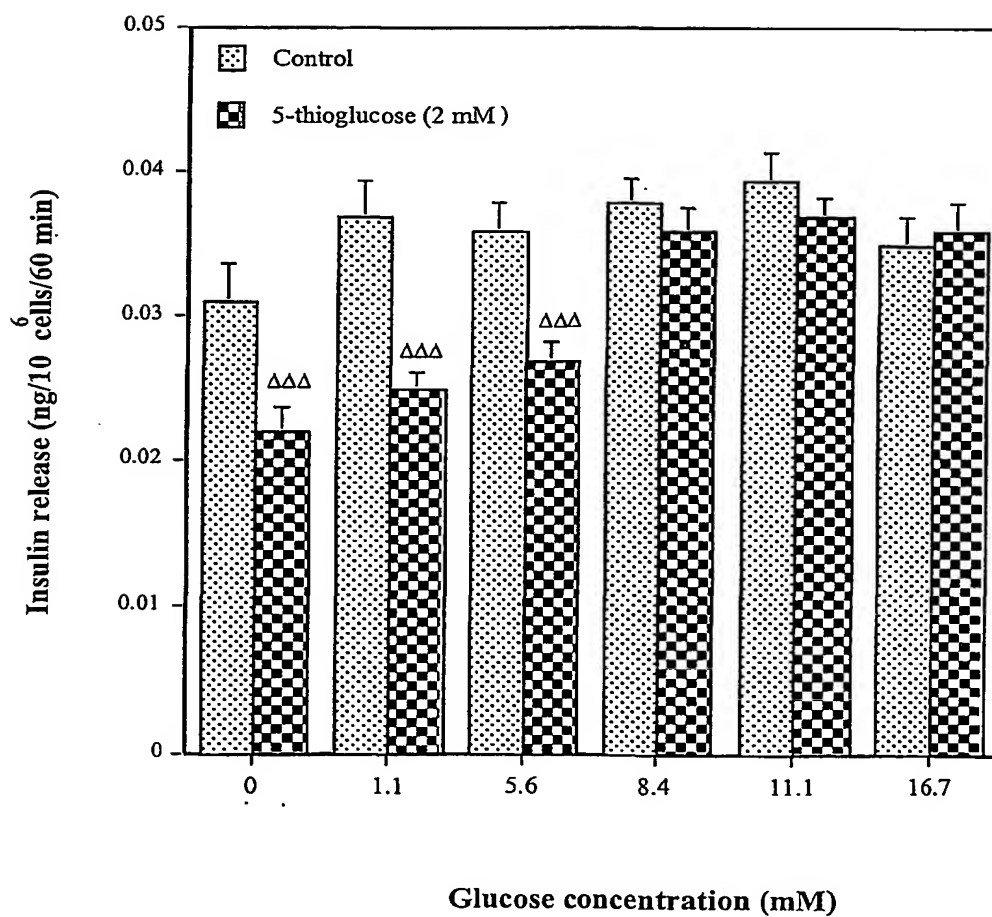
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Figure 20



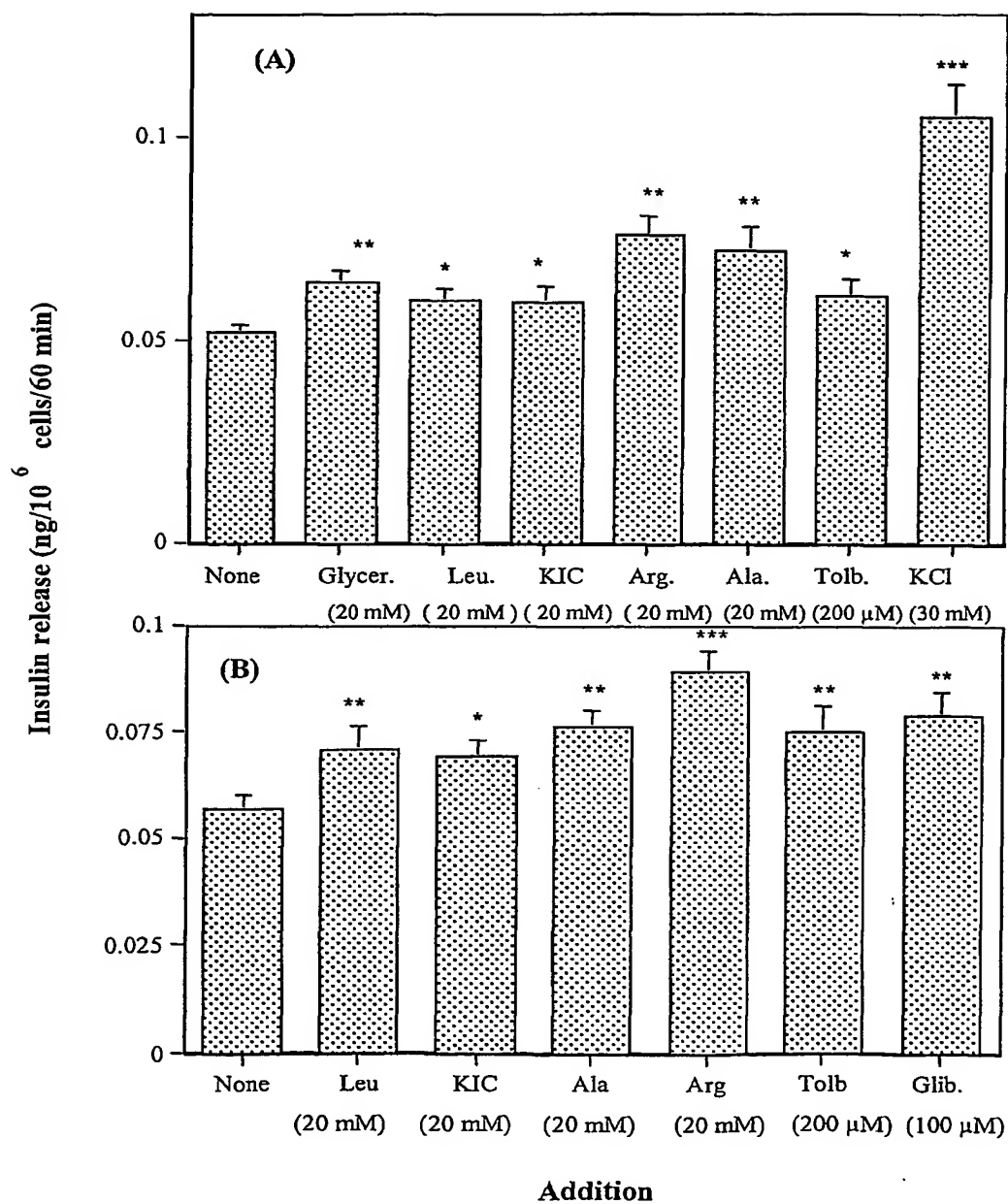
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Figure 21



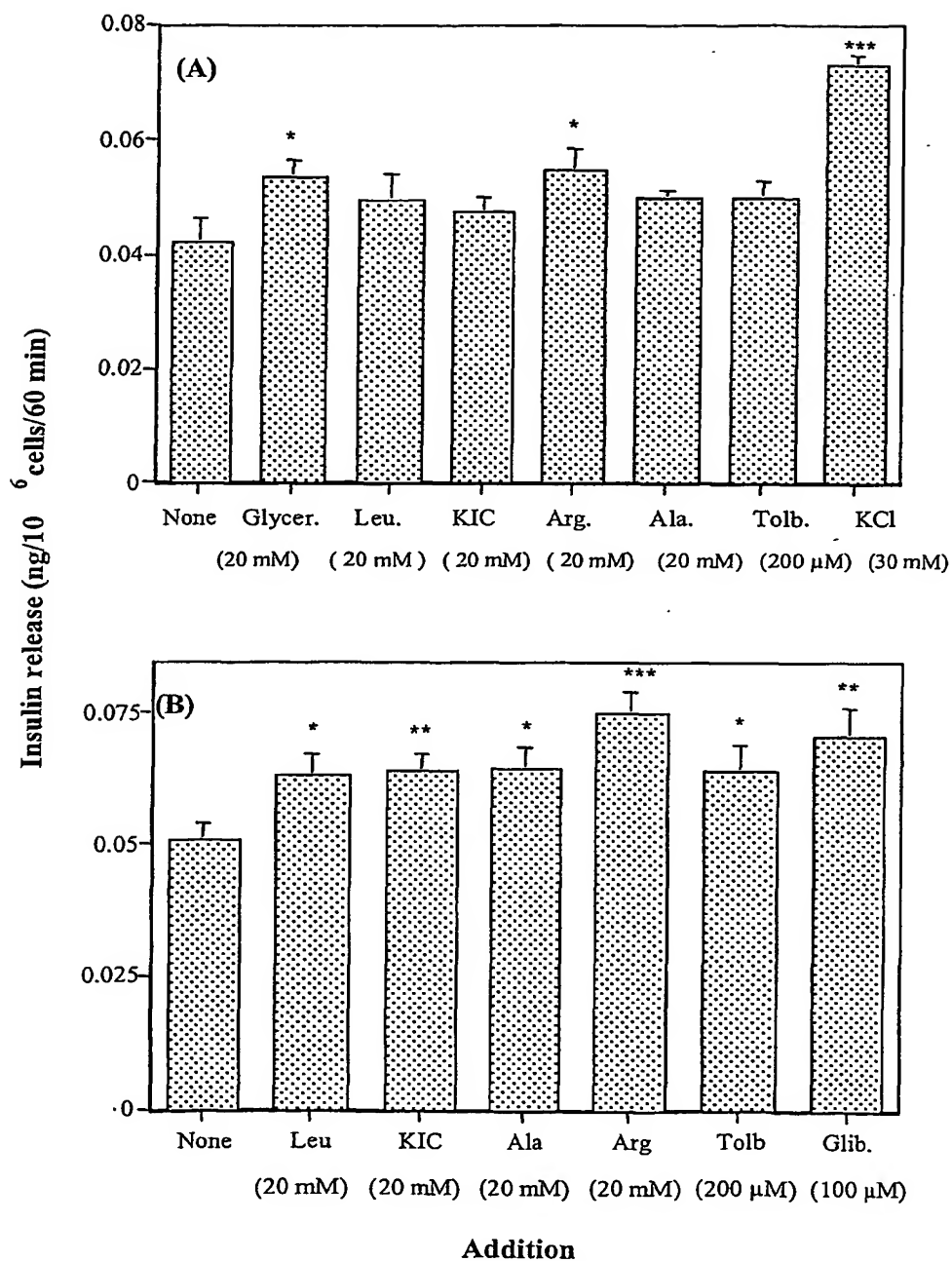
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Figure 22



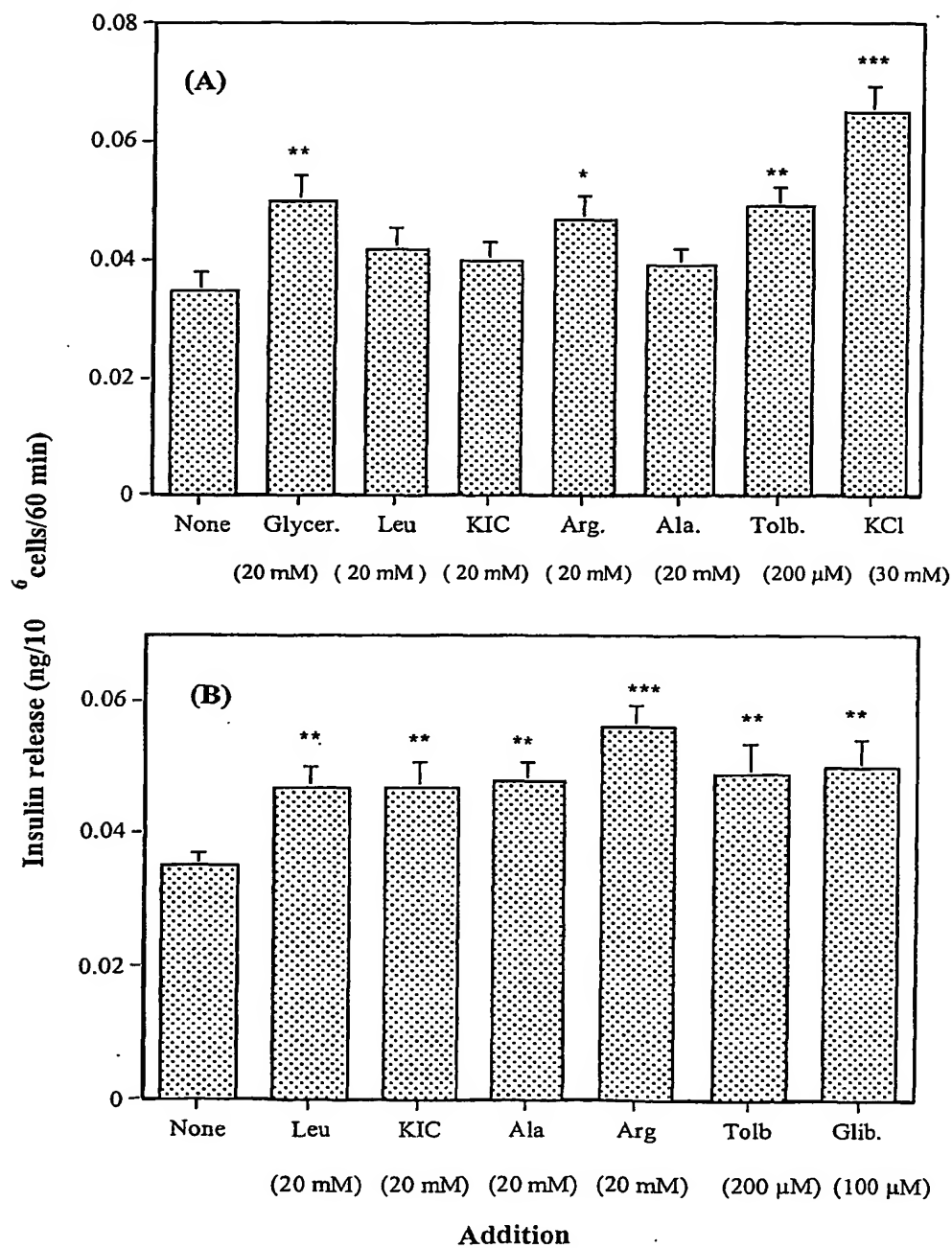
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Figure 23



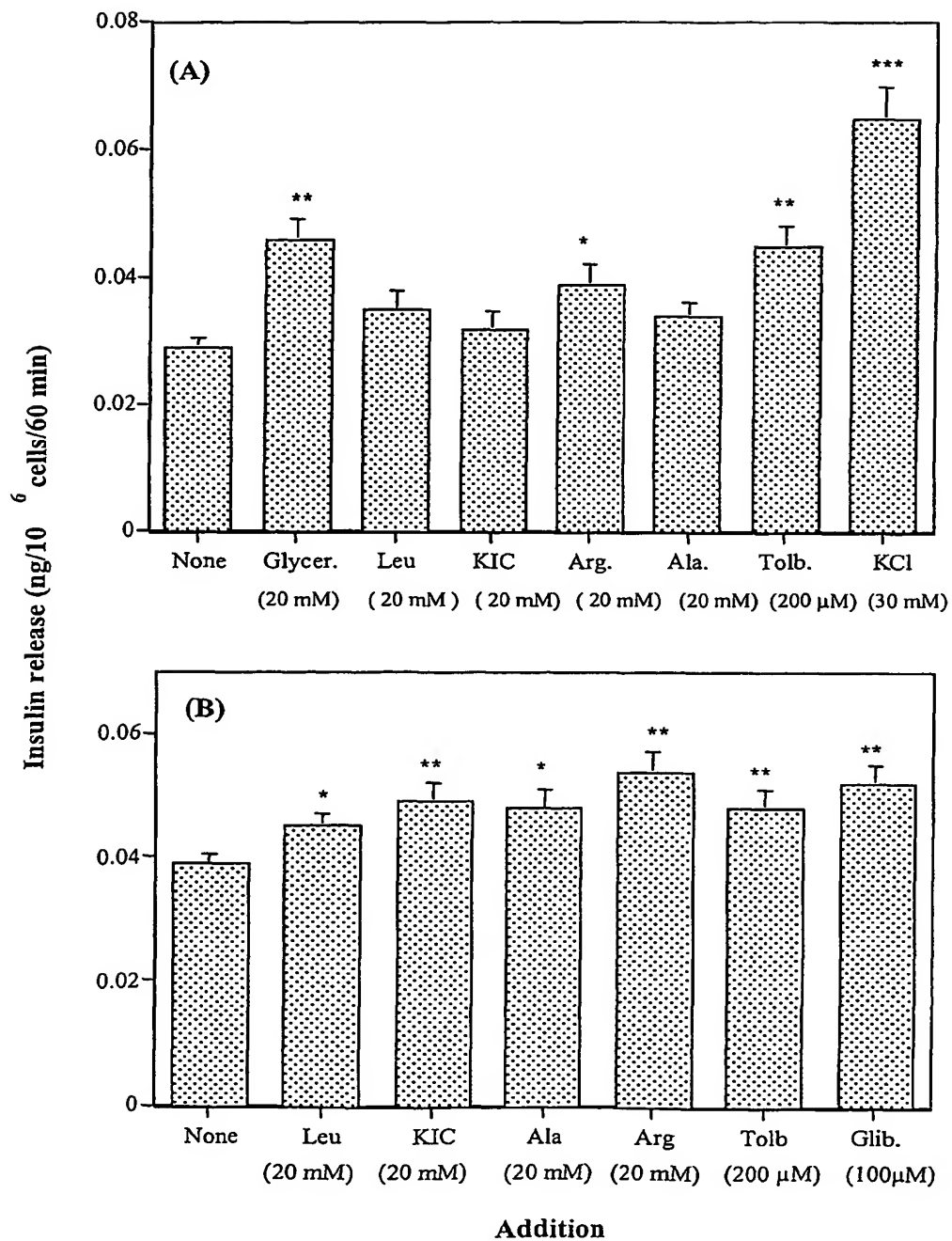
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Figure 24



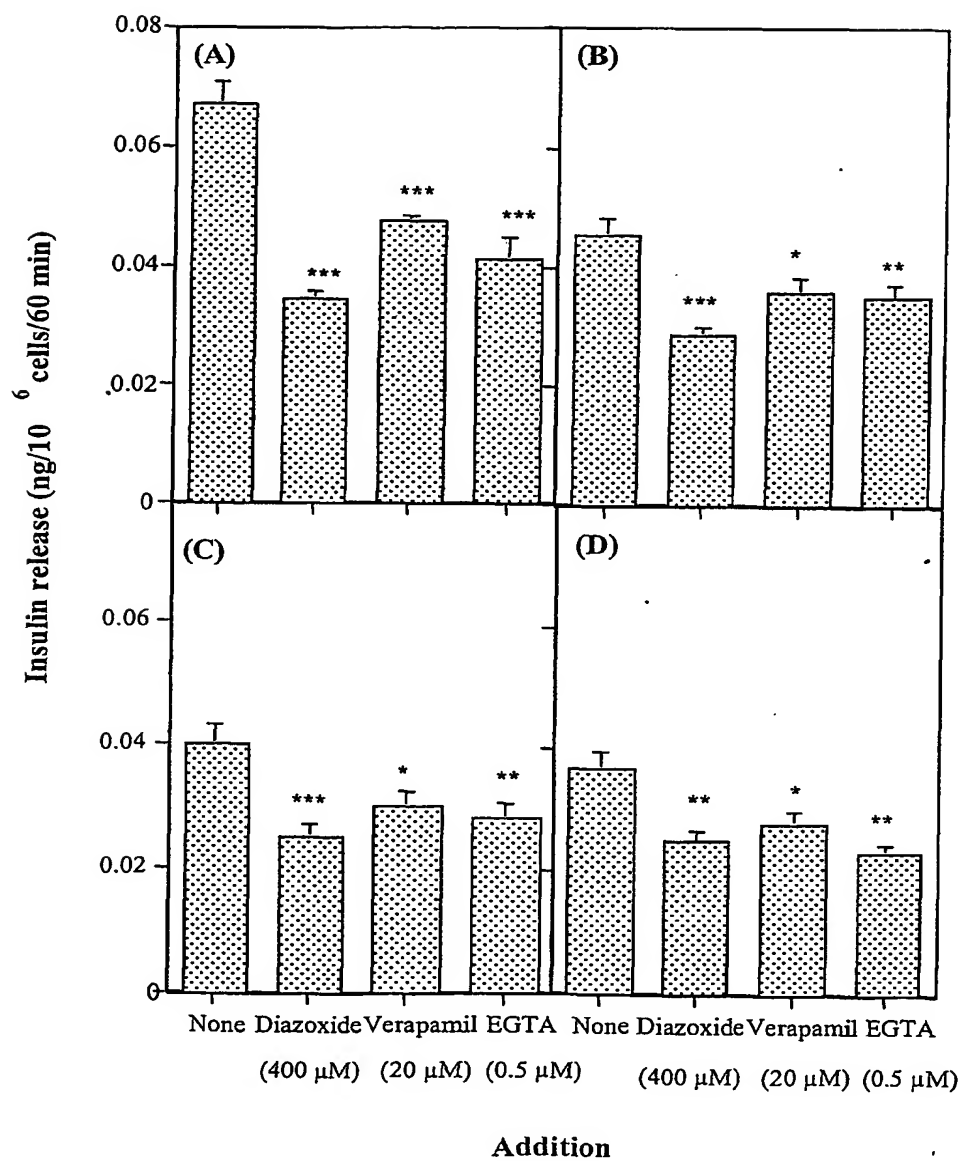
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Figure 25



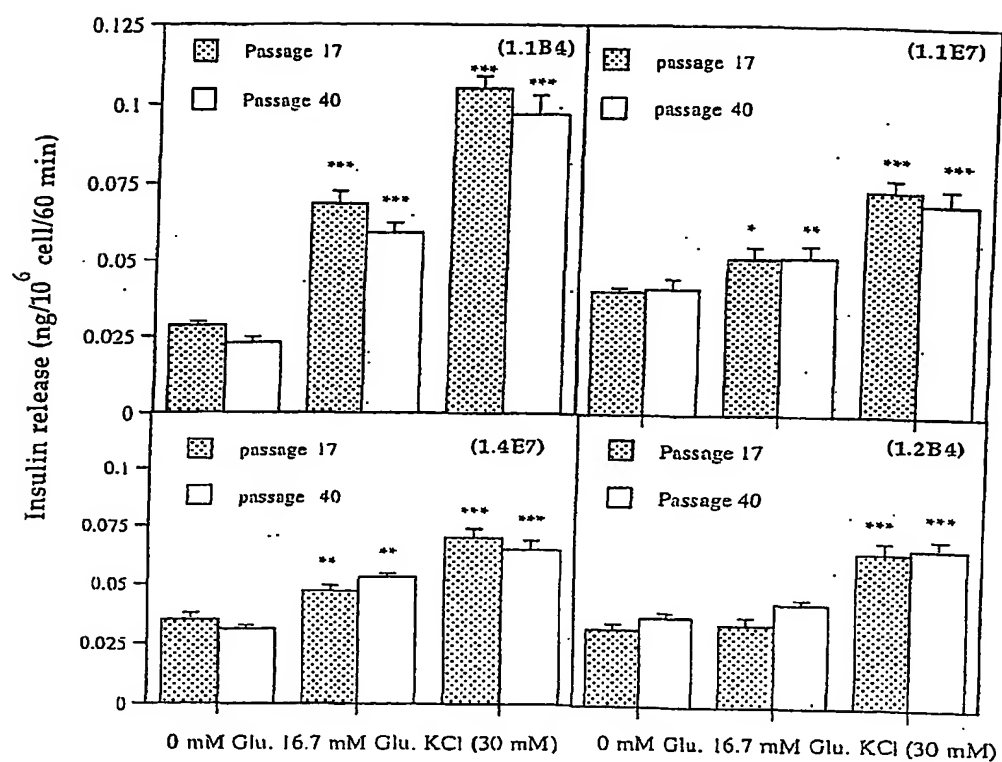
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Figure 26



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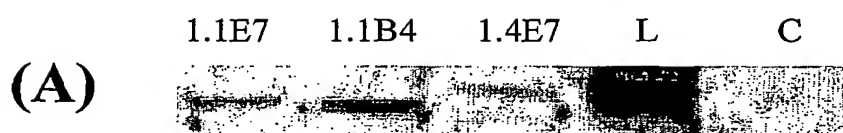
Figure 27



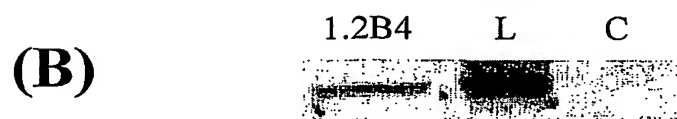
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Figure 28

PANC-1



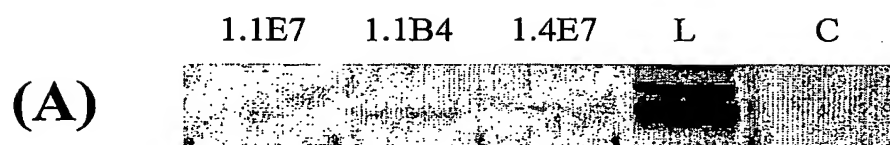
HupT3



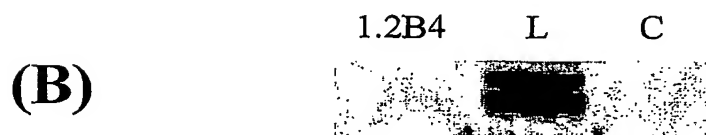
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Figure 29

PANC-1

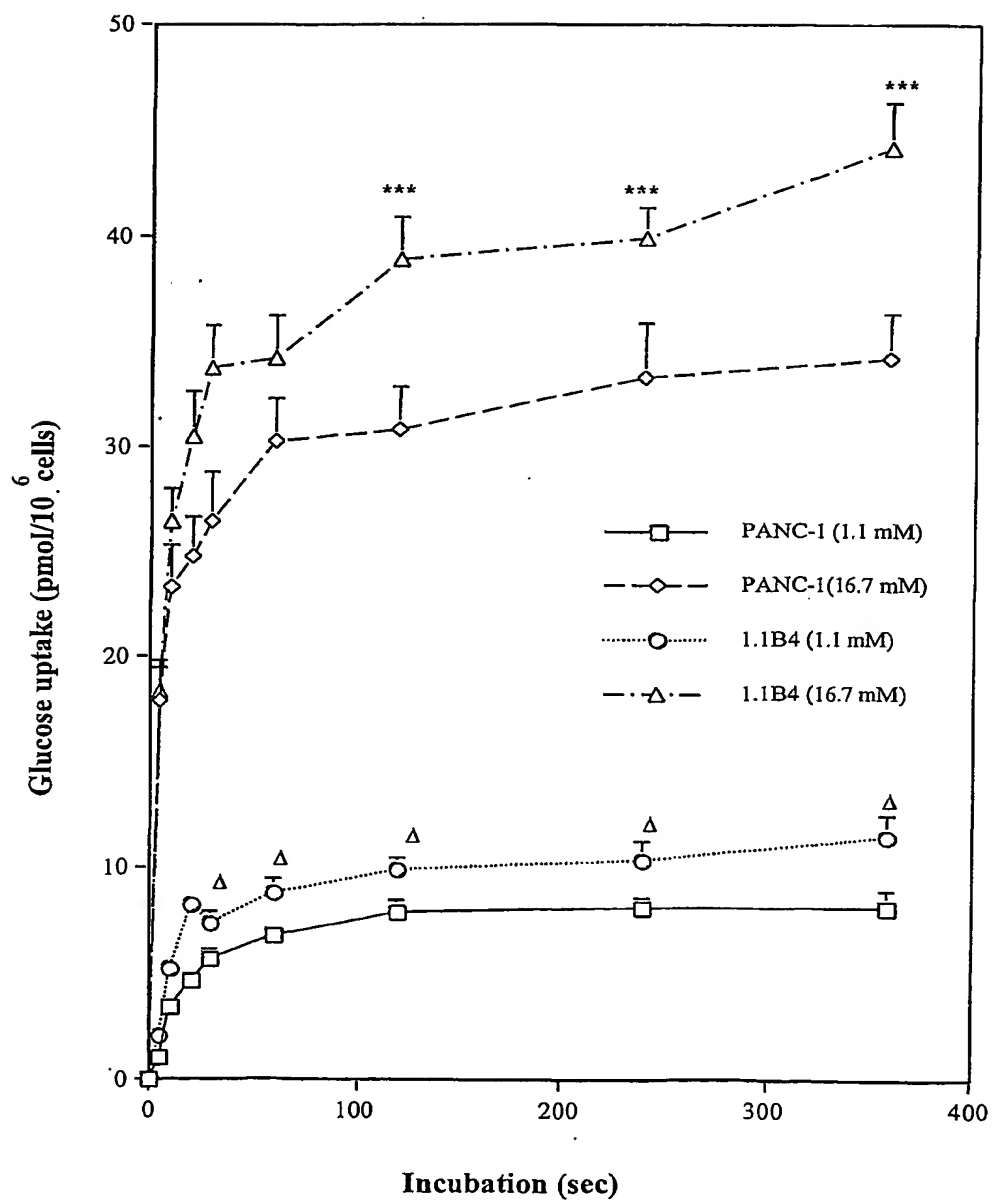


HupT3



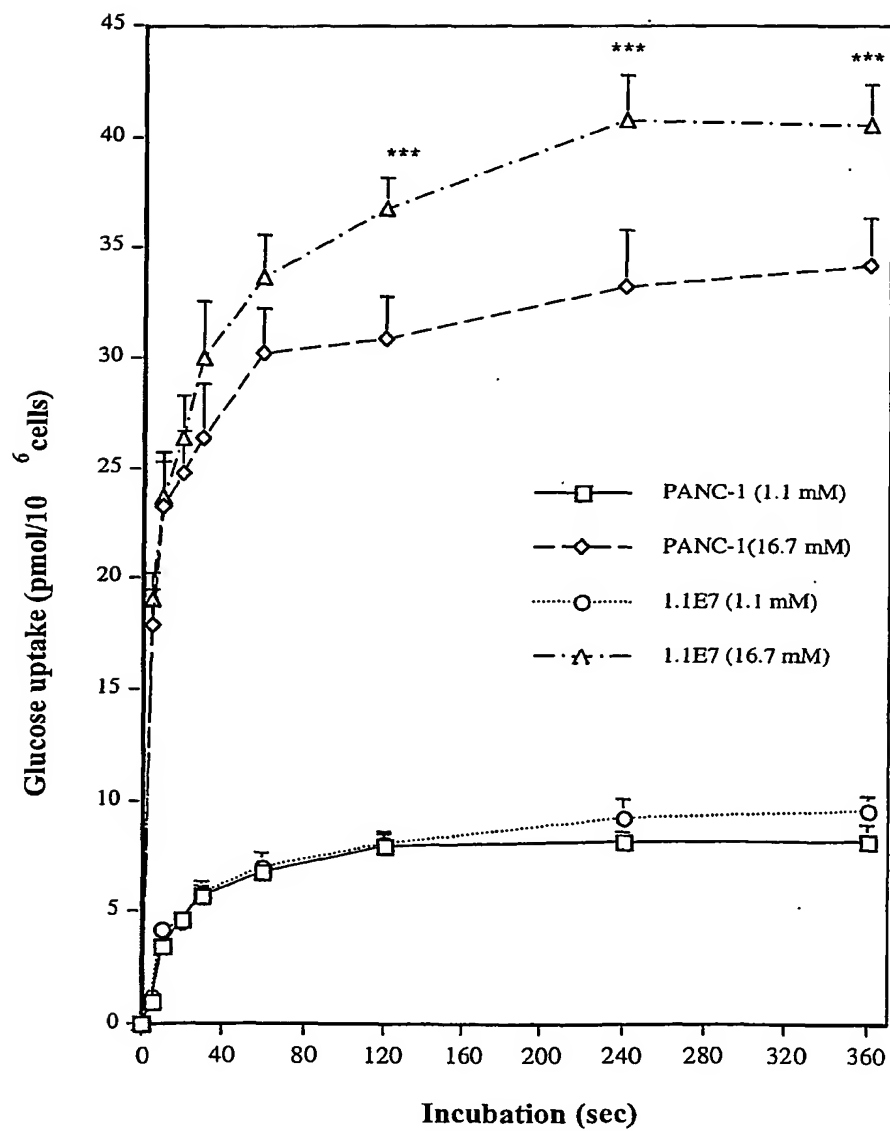
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Figure 30



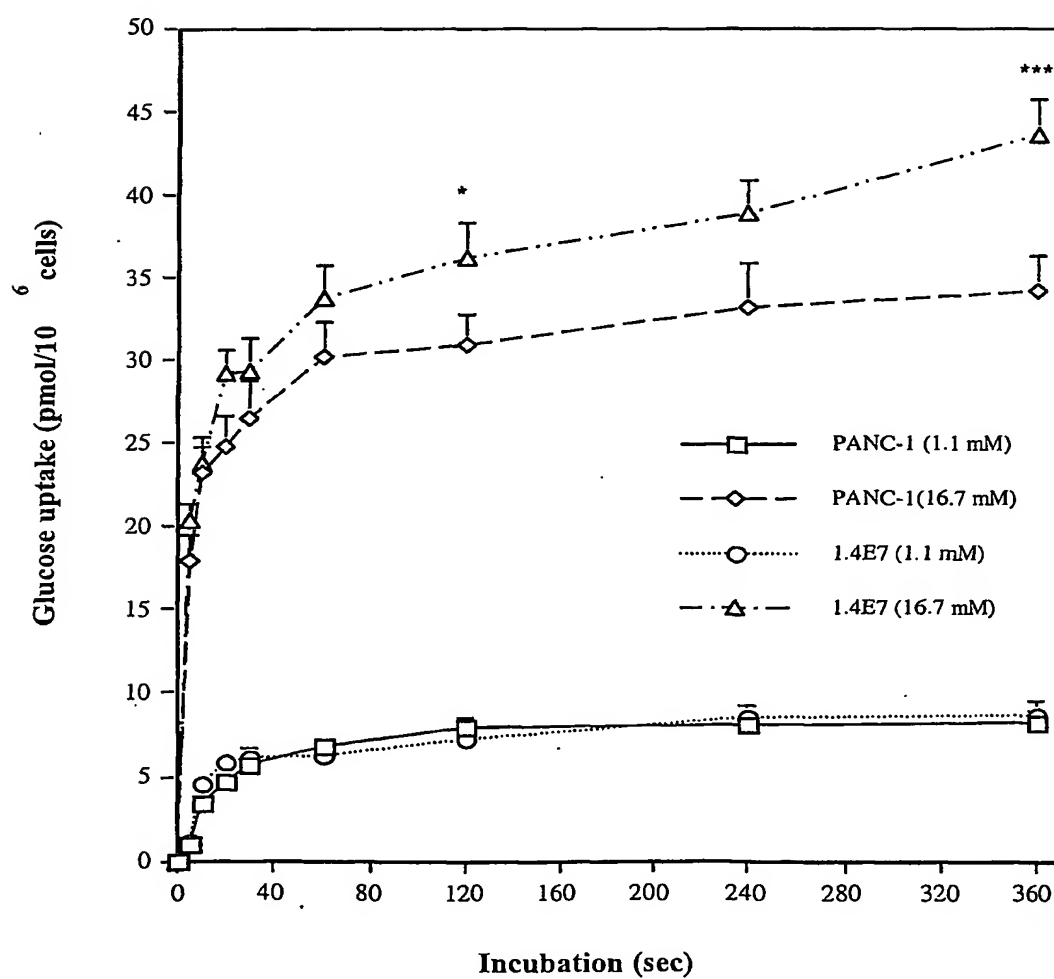
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Figure 31



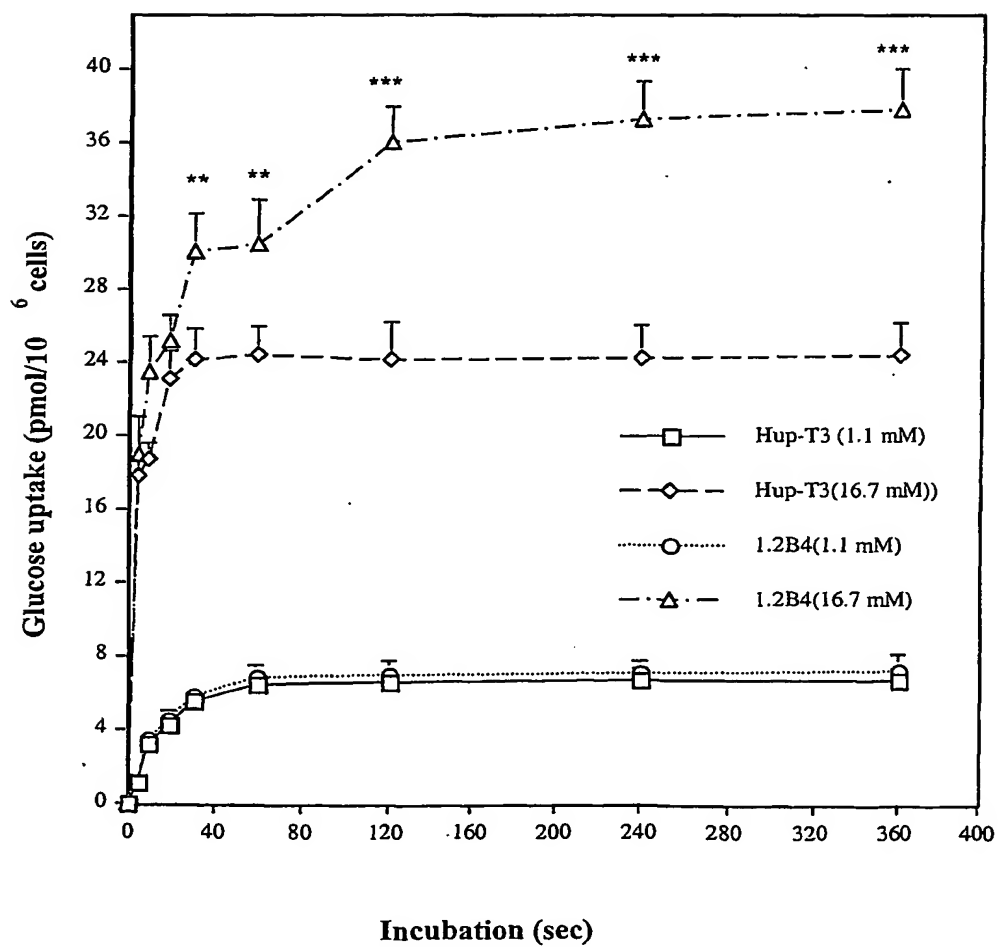
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Figure 32



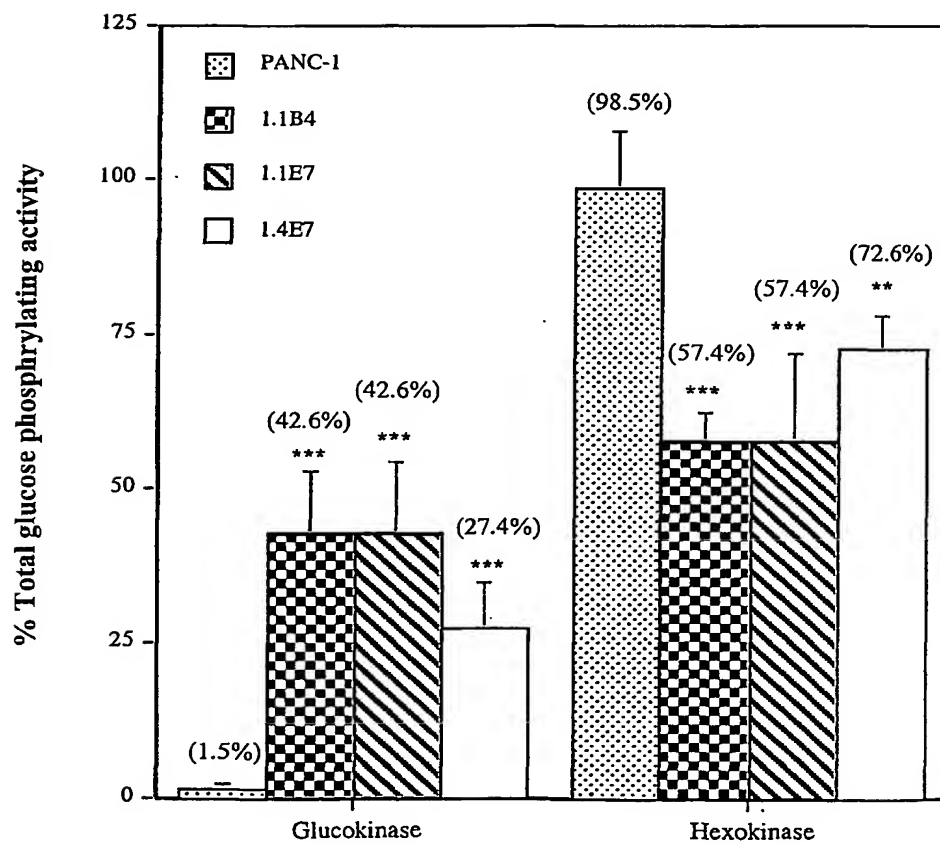
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Figure 33



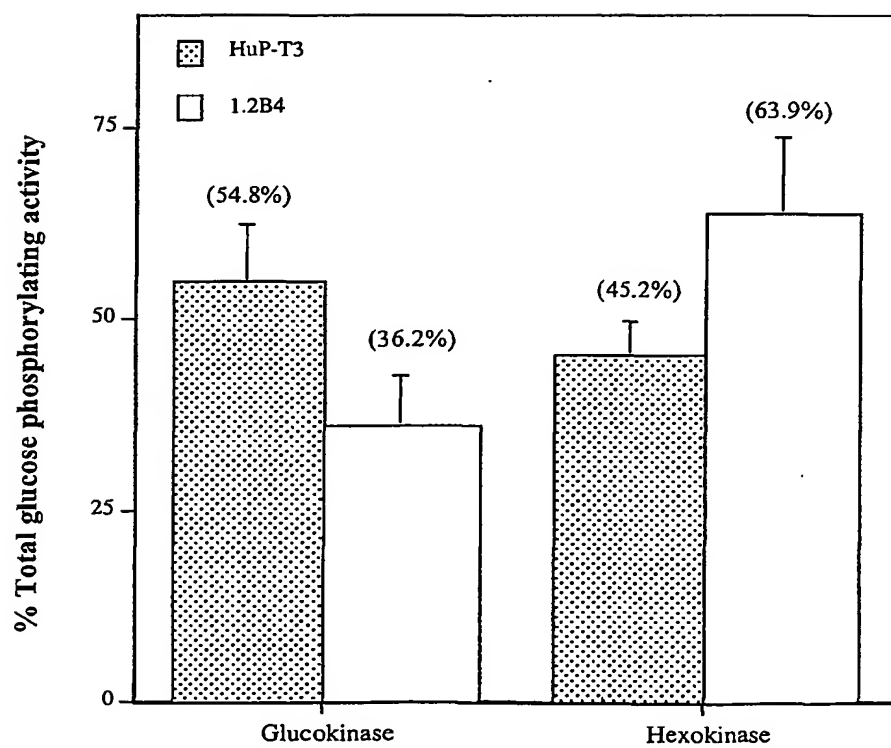
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Figure 34



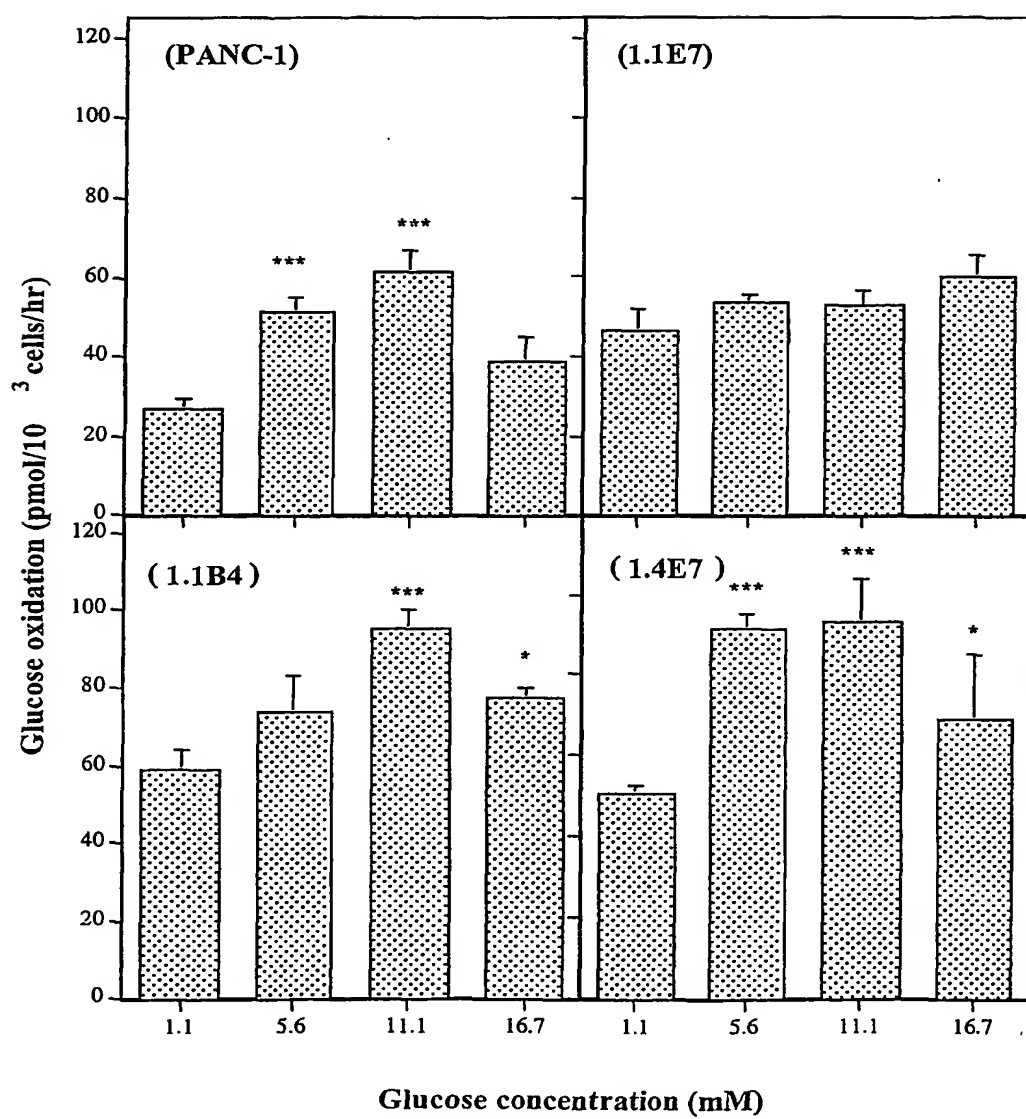
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Figure 35



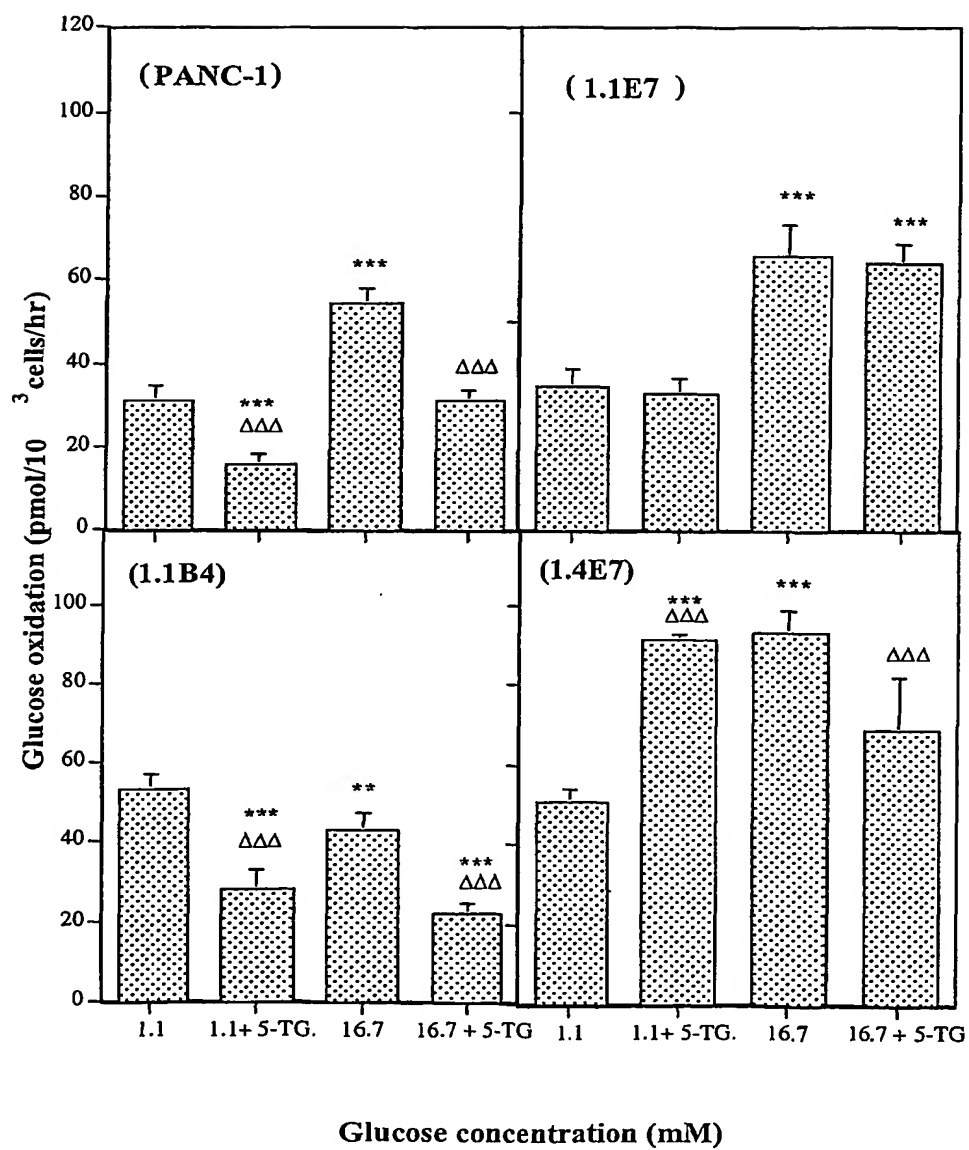
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Figure 36



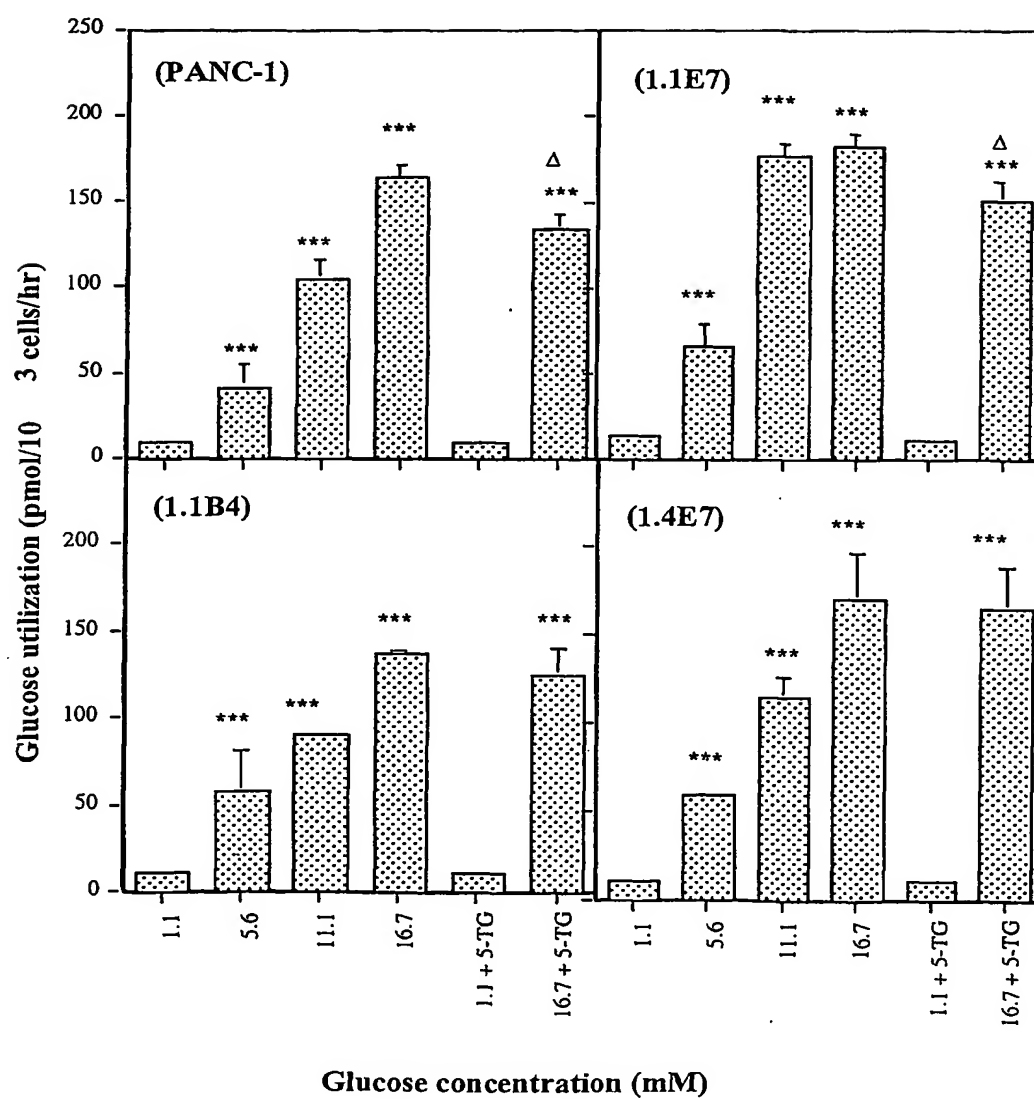
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Figure 37



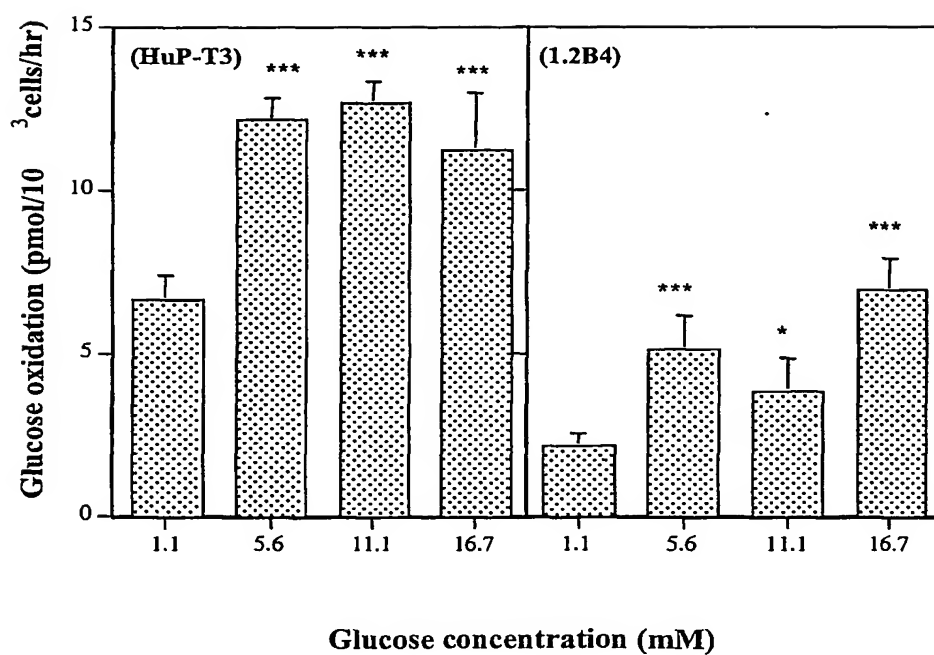
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Figure 38



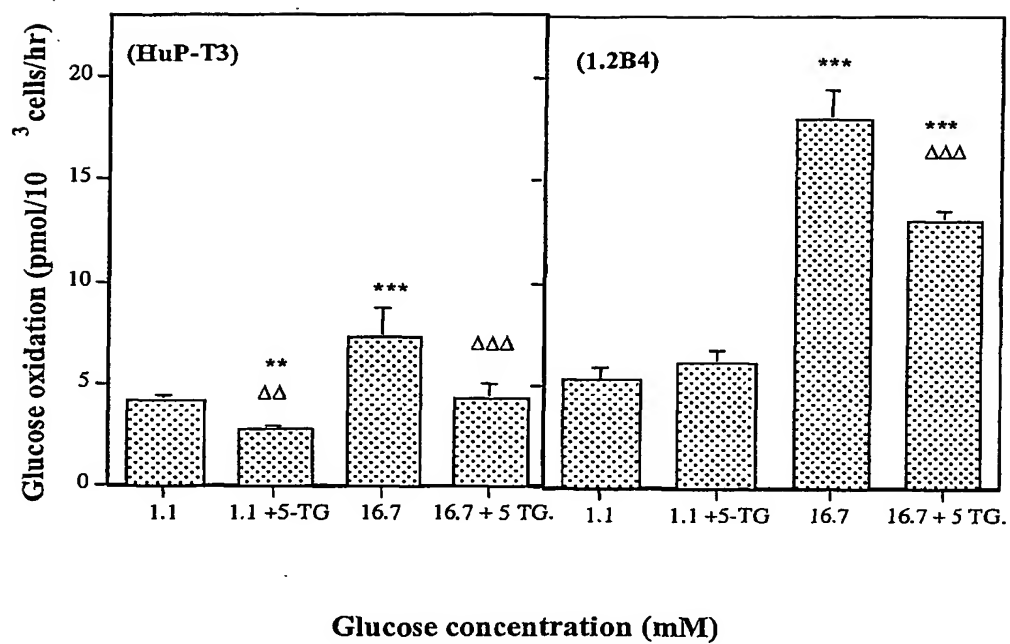
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Figure 39



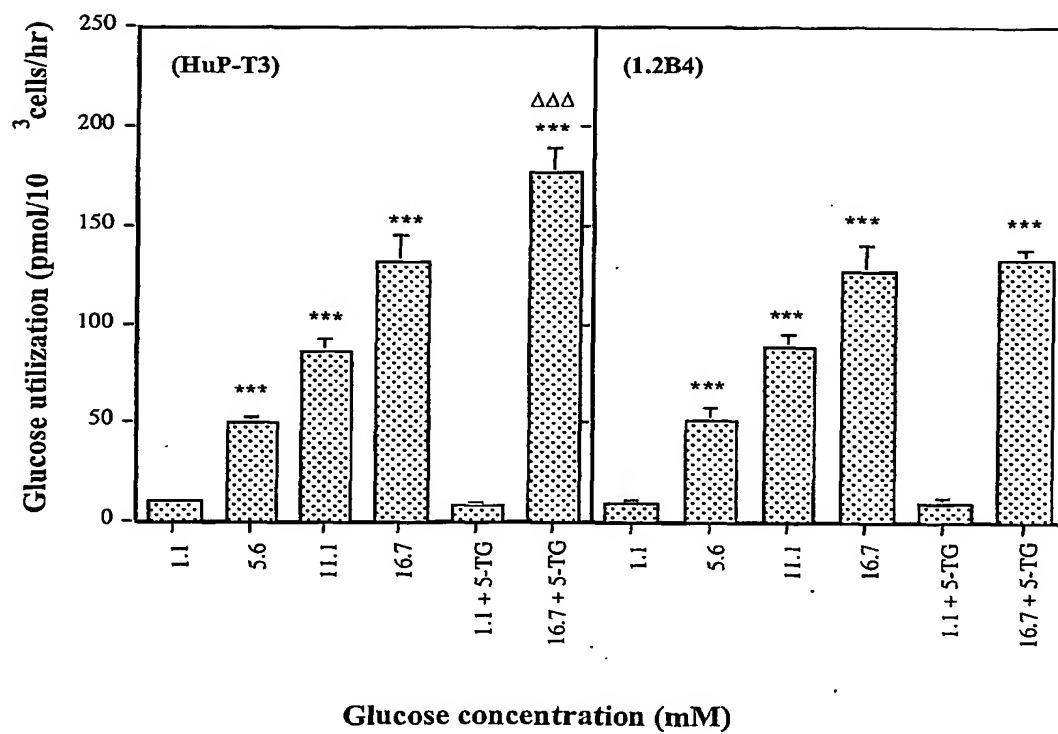
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Figure 40



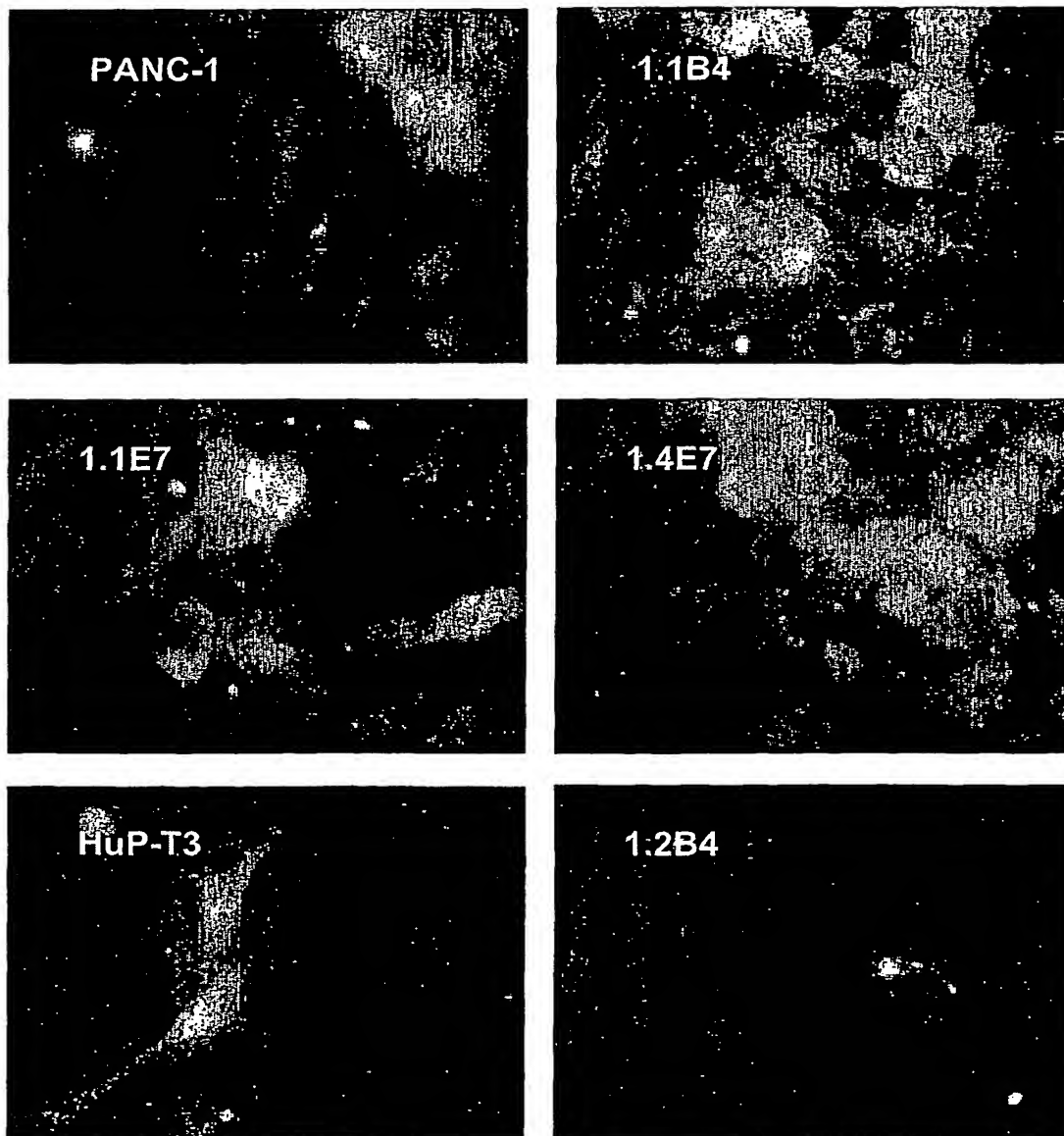
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Figure 41



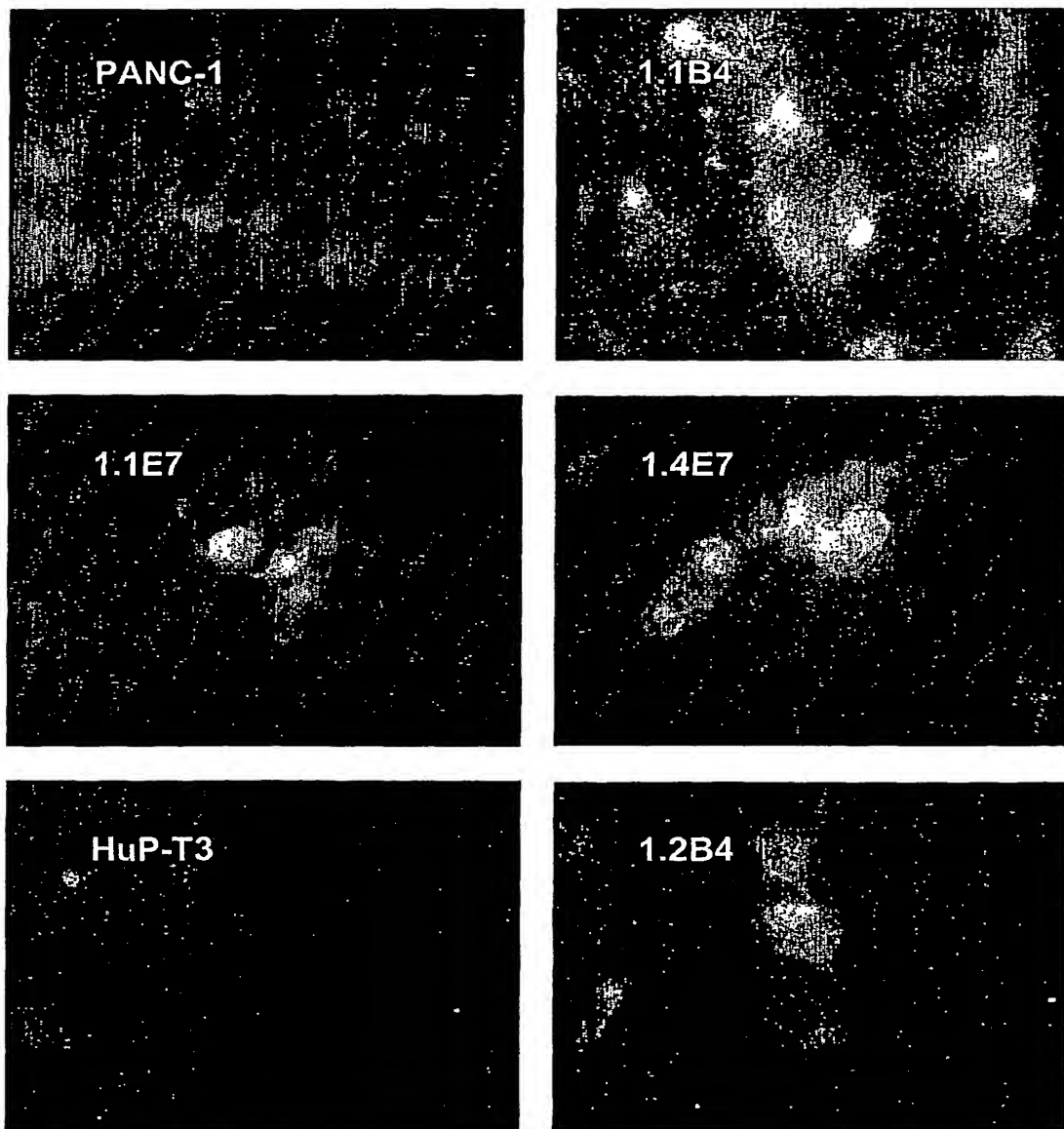
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Figure 42



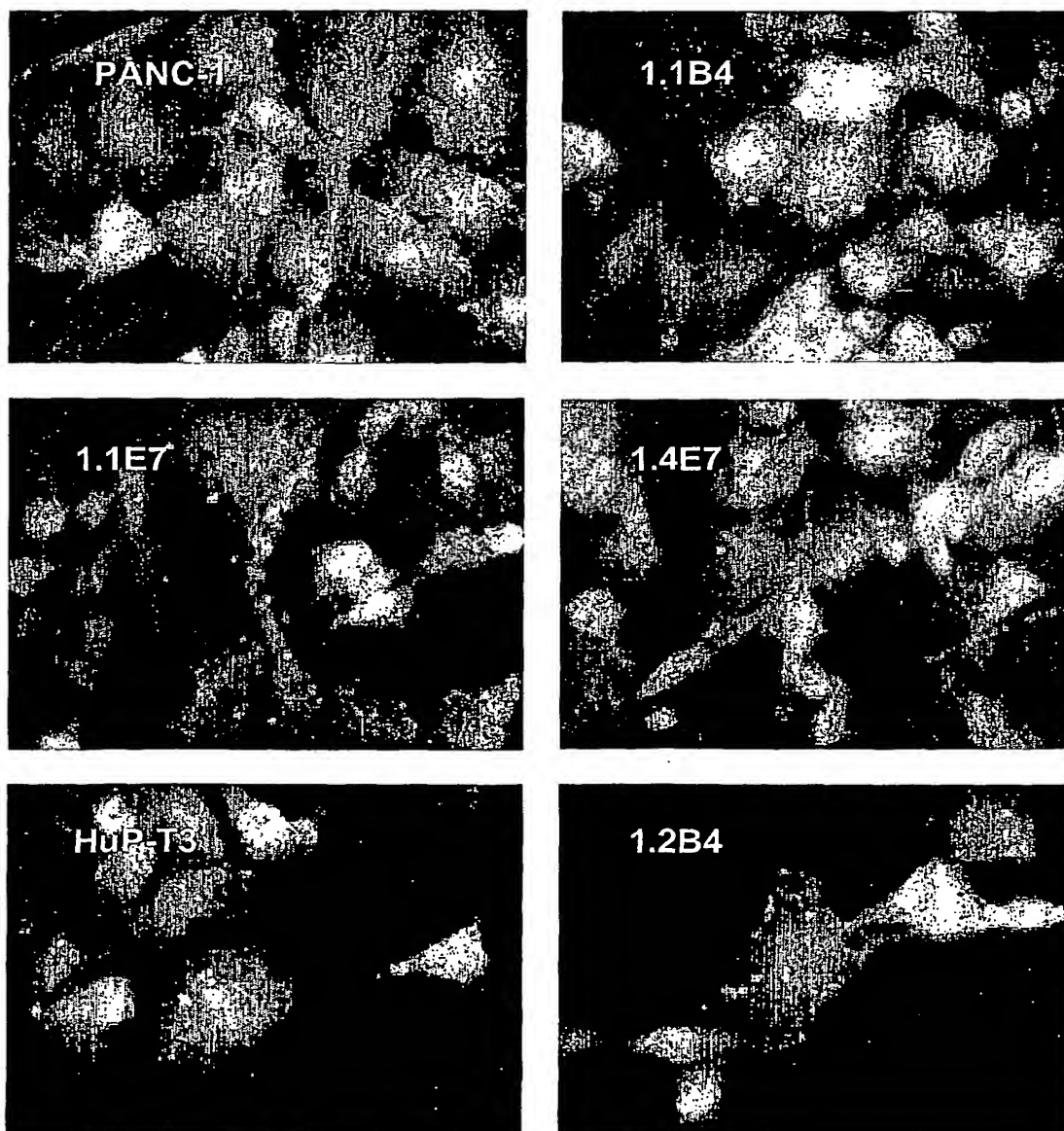
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Figure 43



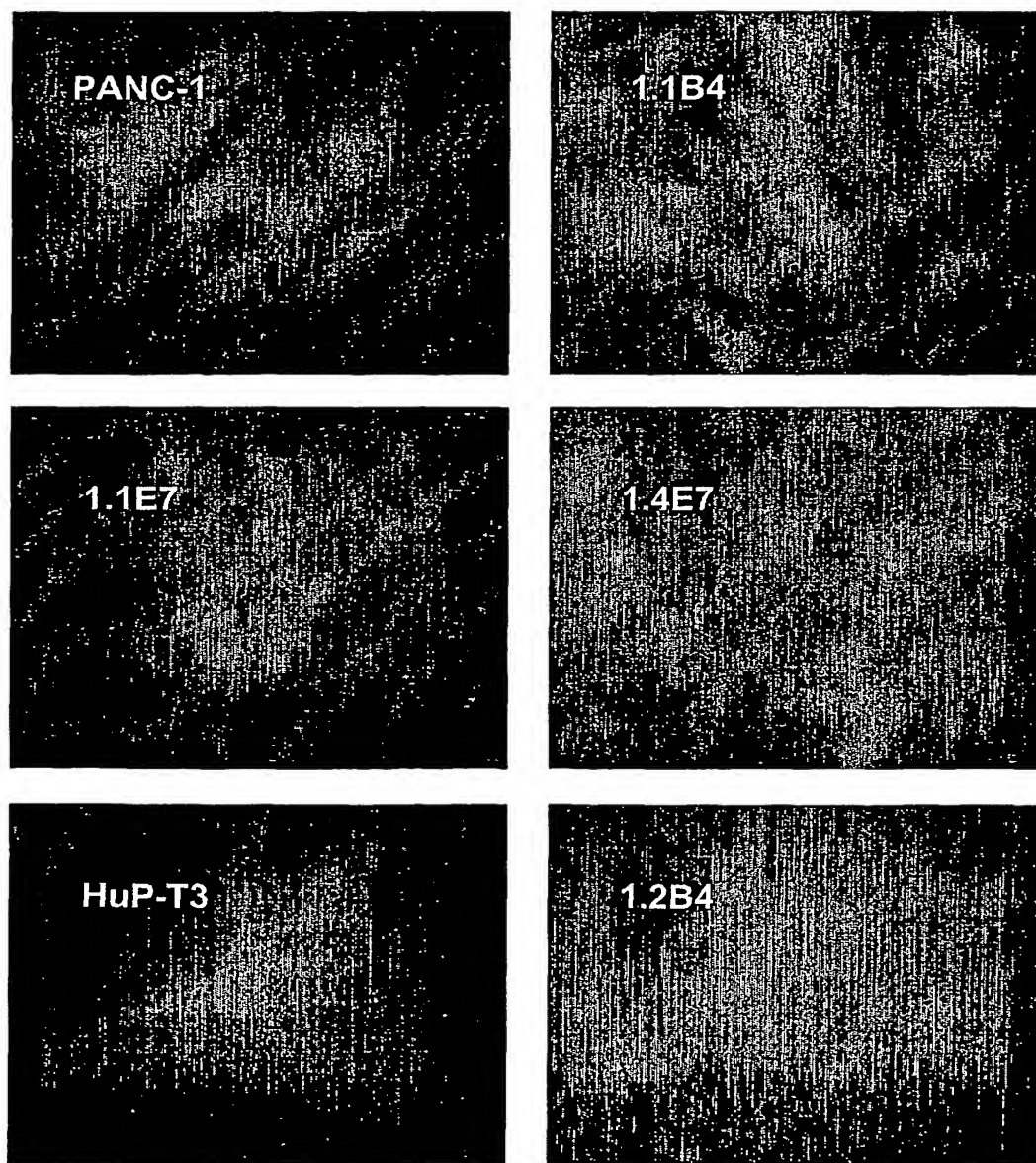
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Figure 44



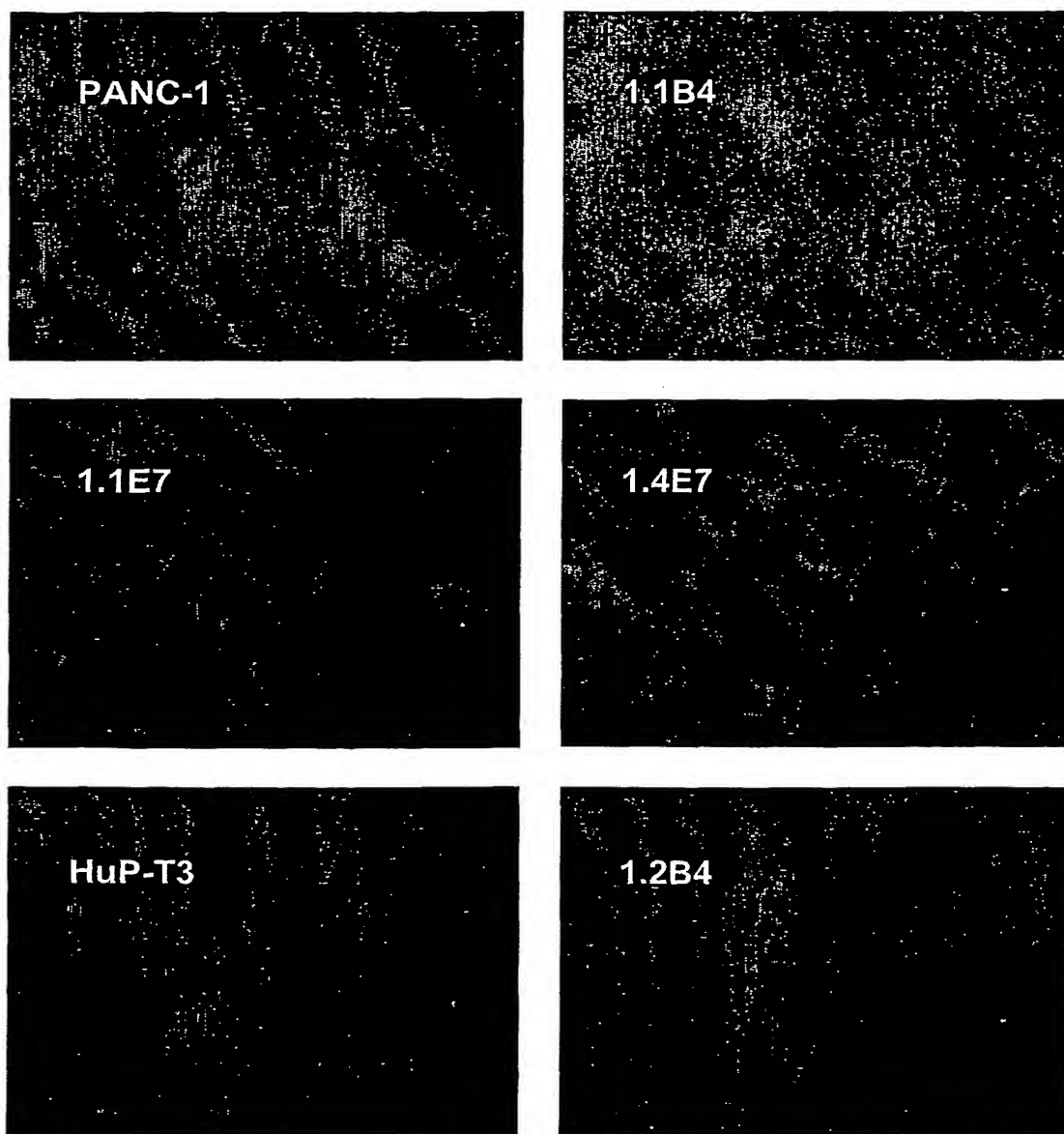
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Figure 45



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Figure 46



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Figure 47**Table 1: Details regarding human islet preparations.**

Islet Preparations	Date Obtained	Donor	Viability In Culture	Fusions Carried Out
1	13/6/97	25 year old: male	67%	14/6/97
2	17/10/97	30 year old: male	39%	18/10/97
3	3/12/97	28 year old: male	71%	4/12/97

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Figure 48

Table2: 3-0-Methyl-D-[1-³H] glucose uptake kinetics of PANC-1, HuP-T3 and human islet-derived insulin-secreting cell lines.

Cell type	Initial velocity ($\mu\text{mol}/10^6 \text{ cell/sec.}$)	
	1.1mM	16.7mM
HuP-T3	0.37 ± 0.03	1.95 ± 0.13
1.2B4	0.39 ± 0.03	$2.54 \pm 0.19^*$
PANC-1	0.45 ± 0.05	2.09 ± 0.21
1.1B4	$0.60 \pm 0.03^*$	$3.05 \pm 0.29^*$
1.1E7	0.47 ± 0.04	$2.88 \pm 0.24^*$
1.4E7	0.51 ± 0.04	$2.70 \pm 0.19^*$

Initial velocities were calculated from the linear uptake phase between 0 and 10 sec. Values are means \pm sem for 3-4 separate experiments. Velocity values were consistently greater at 16.7 versus 1.1mM glucose ($p < 0.001$).

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Figure 49

Table 3: Half maximal equilibration time of 3-0-methyl-D-[1-³H]glucose uptake of kinetics in PANC-1, HuP-T3 and human islet-derived insulin-secreting cell lines.

Cell type	Half maximal equilibration time (sec.)	
	1.1mM	16.7mM
HuP-T3	13.75±1.70	6.00±0.52
1.2B4	10.60±2.13	4.95±0.45
PANC-1	11.05±1.52	5.50±0.37
1.1B4	9.15±0.72	4.13±0.36*
1.1E7	10.85±1.50	4.85±0.51
1.4E7	10.25±1.30	5.00±0.38

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Figure 50

Table 4: Relative metabolic flux through oxidative glucose metabolism in HuP-T3, PANC-1 and human islet-derived insulin-secreting cell lines incubated in 1.1 or 16.7 mmol/l glucose in the absence and presence of 5-thioglucoase (2mmol/l)

Cell type	Glucose oxidation/utilization (ratio)			
	1.1 mM	16.7 mM	1.1 mM + TG	16.7 mM + TG
HuP-T3	0.39±0.04	0.06±0.01	0.31±0.02	0.03±0.01
1.2B4	0.56±0.04*	0.14±0.02**	0.62±0.03***	0.10±0.02*
PANC-1	3.00±0.12	0.33±0.02	1.66±0.15 $\Delta\Delta\Delta$	0.23±0.01 $\Delta\Delta$
1.1B4	5.34±0.17***	0.39±0.02*	2.94±0.21*** $\Delta\Delta\Delta$	0.32±0.03*
1.1E7	3.96±0.26***	0.38±0.01*	3.04±0.19*** Δ	0.28±0.02 Δ
1.4E7	3.36±0.21**	0.38±0.01*	5.12±0.22*** $\Delta\Delta$	0.30±0.02*

Values are mean \pm sem (n=4). *p<0.05, **p<0.01, ***p<0.001 compared with parental cells (HuP-T3 or PANC-1). Δ p<0.05, $\Delta\Delta$ p<0.01, $\Delta\Delta\Delta$ p<0.001 compared with same glucose concentration in absence of 5-TG.

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Figure 51**Table 5: Summary of immunocytochemical investigations of functional proteins in parental and novel human islet cell lines.**

Cell lines	Glucokinase	Insulin	IAPP	Glucagon	Somostatin
HuP-T3	+	-	++	-	-
1.2B4	++	+	++	-	-
PANC-1	+	-	++	-	-
1.1B4	+++	+++	+++	-	-
1.1E7	+++	+	+++	-	-
1.4E7	+++	++	+++	-	-

Intensity of staining: Lacking (-); Weak (+); Moderate (++); Strong (+++). Original data were presented in Figures 42 to 46.

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Figure 52

Cell line	Insulin content	Glucose response	Response to other stimuli	GLUT-1 expression transport	Glucose stimulated transport	GK expression	% of GK	Improve-ment with 5-thiogluco- se	Glucose oxi./ Utilisation ratio
HuP-T3	-	-	-	-	-	-	++	+	+
1.2B4	++	+	++	+	++	++	++	++	++
PANC-1	-	-	-	-	-	-	+	-	++
1.1B4	++	+++	+++	+++	+++	++	++	++	+++
1.1E7	++	++	++	++	++	++	++	+	+++
1.4E7	++	++	++	++	++	++	++	++	+++

Table 6: Summary of functional characteristics of parental and novel human islet cell lines.

Strength of features; Lacking (-); Weak, certain condition only (+); Moderate (++); Strong (+++). Original data are presented in figures 9 to 41

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Figure 53

Table 7: Identity profile results of 1.4E7

LOCUS	CHROMOSOME LOCATION	SAMPLE # 1.4E7, P15
D3S1358	3p	17,17
vWA	12p12-pter	15,15
FGA	4q28	21,21
Amelogenin	X:p22.1-22.3/Y:p11.2	X,X
D8S1179	8	14,15
D21S11	21	28,28
D18S51	18q21.3	12,12
D5S818	5q21-31	11,13
D13S317	13q22-31	11,11
D7S820	7q11.21-22	8,8
D16S539	16q24-qter	11,11
THO1	11p15.5	7,8
TPOX	2p23-2per	8,11
CSF1PO	5q33.3-34	10,12

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Figure 54

Table 8: Identity profile results 1.1E7

LOCUS	CHROMOSOME LOCATION	SAMPLE# 1.1E7, P14
D3S1358	3p	17,17
vWA	12p12-pter	15,15
FGA	4q28	21,21
Amelogenin	X: p22.1-22.3/Y: p11.2	X,X
D8S1179	8	14,15
D21S11	21	28,28
D18S51	18q21.3	12,12
D5S818	5q21-31	11,13
D13S317	13q22-31	11,11
D7S820	7q11.21-22	8,8
D16S539	16q24-qter	11,11
THO1	11p15.5	7,8
TPOX	2p23-2per	8,11
CSF1PO	5q33.3-34	10,12

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Figure 55

Table 9: Identity profile results of 1.1B4

LOCUS	CHROMOSOME LOCATION	SAMPLE# 1.1B4 P24
D3S1358	3p	17,17
vWA	12p12-pter	15,15
FGA	4q28	21,21
Amelogenin	X: p22.1-22.3/Y: p11.2	X,X
D8S1179	8	14,15
D21S11	21	28,28
D18S51	18q21.3	12,12
D5S818	5q21-31	11,13
D13S317	13q22-31	11,11
D7S820	7q11.21-22	8,8
D16S539	16q24-qter	11,11
THO1	11p15.5	7,8
TPOX	2p23-2per	8,11
CSF1PO	5q33.3-34	10,12

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Figure 56

Table 10: Identity profile results of 1.2B4

LOCUS	CHROMOSOME LOCATION	SAMPLE# 1.2B4 P22
D3S1358	3p	15,16
vWA	12p12-pter	18,18
FGA	4q28	21,22
Amelogenin	X: p22.1-22.3/Y: p11.2	X,Y
D8S1179	8	15,15
D21S11	21	28,30
D18S51	18q21.3	16,17
D5S818	5q21-31	10,14
D13S317	13q22-31	9,11
D7S820	7q11.21-22	12,12
D16S539	16q24-qter	10,13
THO1	11p15.5	9,9
TPOX	2p23-2per	8,12
CSF1PO	5q33.3-34	10,10

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